# RoC
# Background Document
# for
# Diazoaminobenzene

November 22, 2002

# FOREWORD

The Report on Carcinogens (RoC) is prepared in response to Section 301 of the Public Health Service Act as amended. The RoC contains a list of all substances (i) that either are known to be human carcinogens or may reasonably be anticipated to be human carcinogens; and (ii) to which a significant number of persons residing in the United States are exposed. The Secretary, Department of Health and Human Services (DHHS) has delegated responsibility for preparation of the RoC to the National Toxicology Program (NTP) who prepares the Report with assistance from other Federal health and regulatory agencies and non-government institutions.

Nominations for listing in or delisting from the RoC are reviewed by a formal process that includes a multi-phased, scientific peer review and multiple opportunities for public comment. The review groups evaluate each nomination according to specific RoC listing criteria. This Background Document was prepared to assist in the review of the nomination of diazoaminobenzene. The scientific information in this document comes from publicly available, peer reviewed sources. Any interpretive conclusions, comments or statistical calculations, etc made by the authors of this document that are not contained in the original citation are identified in brackets [ ]. If any member(s) of the scientific peer review groups feel this Background Document does not adequately capture and present the relevant information they will be asked to write a commentary for this Background Document that will be included as an addendum to the document. In addition, a meeting summary that contains a brief discussion of the respective review group's review and recommendation for the nomination will be added to the Background Document, also as an addendum.

A detailed description of the RoC nomination review process and a list of all nominations under consideration for listing in or delisting from the RoC can be obtained by accessing the NTP Home Page at http://ntp-server.niehs.nih.gov. The most recent RoC, the 9th Edition, was published in May, 2000 and may be obtained by contacting the NIEHS Environmental Health Information Service (EHIS) at http://ehis.niehs.nih.gov (800-315-3010).

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## Criteria for Listing Agents, Substances or Mixtures in the Report on Carcinogens

**U.S. Department of Health and Human Services**
**National Toxicology Program**

### Known to be Human Carcinogens:

There is sufficient evidence of carcinogenicity from studies in humans, which indicates a causal relationship between exposure to the agent, substance or mixture and human cancer.

### Reasonably Anticipated to be Human Carcinogens:

There is limited evidence of carcinogenicity from studies in humans, which indicates that causal interpretation is credible but that alternative explanations such as chance, bias or confounding factors could not adequately be excluded; or

There is sufficient evidence of carcinogenicity from studies in experimental animals which indicates there is an increased incidence of malignant and/or a combination of malignant and benign tumors: (1) in multiple species, or at multiple tissue sites, or (2) by multiple routes of exposure, or (3) to an unusual degree with regard to incidence, site or type of tumor or age at onset; or

There is less than sufficient evidence of carcinogenicity in humans or laboratory animals, however; the agent, substance or mixture belongs to a well defined, structurally-related class of substances whose members are listed in a previous Report on Carcinogens as either a *known to be human carcinogen, or reasonably anticipated to be human carcinogen* or there is convincing relevant information that the agent acts through mechanisms indicating it would likely cause cancer in humans.

Conclusions regarding carcinogenicity in humans or experimental animals are based on scientific judgment, with consideration given to all relevant information. Relevant information includes, but is not limited to dose response, route of exposure, chemical structure, metabolism, pharmacokinetics, sensitive sub populations, genetic effects, or other data relating to mechanism of action or factors that may be unique to a given substance. For example, there may be substances for which there is evidence of carcinogenicity in laboratory animals but there are compelling data indicating that the agent acts through mechanisms which do not operate in humans and would therefore not reasonably be anticipated to cause cancer in humans.

# Executive Summary

**Introduction**
Diazoaminobenzene (DAAB) is a triazene that is used as an intermediate in chemical synthesis, as a complexing agent, and as a polymer additive. DAAB was nominated by the National Institute for Environmental Health Sciences (NIEHS) for possible listing in the Report on Carcinogens based on the results of short-term toxicity and metabolism studies by the National Toxicology Program which found that DAAB is metabolized to the known human carcinogen benzene and to the animal carcinogen aniline.

**Human Exposure**
*Use.* DAAB is used as a chemical intermediate, complexing agent, and polymer additive. It has uses in organic synthesis and manufacture of dyes and insecticides and as a dopant for laser ablation of polymethylmethacrylate. DAAB has been identified as a contaminant in the dyes D&C Red No. 33, FD&C Yellow No. 5 (tartrazine), and FD&C Yellow No. 6; all are permitted for use in drugs and cosmetics, and the latter two are permitted in food.

*Production*. DAAB may be produced by reaction of aniline with isoamyl nitrate or by diazotization of aniline dissolved in hydrochloric acid with sodium nitrite, followed by addition of sodium acetate. No data were found on U.S. production of DAAB, but it was available from seven U.S. suppliers in 2001. U.S. imports of DAAB and *p*-aminoazobenzenedisulfonic acid (combined category) totaled 94,237 lb (42,835 kg) from January through October 2001.

*Environmental Exposure.* Environmental exposure to DAAB may occur from oral consumption of or dermal exposure to dyes and colorants (such as FD&C Yellow No. 5). A study conducted by the National Academy of Sciences in 1977 reported that average daily intakes were 43 mg of FD&C Yellow No. 5 and 37 mg of FD&C Yellow No. 6. Based on these intakes and the maximum allowable levels of DAAB in colorants under U.S. Food and Drug Administration (FDA) regulations, the theoretical maximum daily exposures to DAAB are approximately 1.7 ng for FD&C Yellow No. 5 and 1.5 ng for FD&C Yellow No. 6.

*Occupational exposure*. Occupational exposure to DAAB could occur from its use as a chemical intermediate and polymer additive. DAAB is explosive and is harmful to the respiratory tract, skin, and eyes through inhalation or dermal contact.

*Regulations.* No DAAB-specific regulations were found. The FDA regulates FD&C Yellow No. 5 and FD&C Yellow No. 6 for use as color additives in foods, drugs, and cosmetics and D&C Red No. 33 for use as a color additive in drugs and cosmetics.

**Human Cancer Studies**
No human studies were identified in which exposure to DAAB is specifically mentioned. DAAB is predicted to be a carcinogen because it is metabolized to benzene and aniline. Benzene is classified by the Report on Carcinogens as *known to be a human carcinogen* and by the International Agency for Research on Cancer (IARC) as carcinogenic to

humans (Group 1). Numerous epidemiological studies have shown a causal relationship between benzene exposure and leukemia. Aniline was listed by IARC in 1987 as not classifiable as to its carcinogenicity to humans (Group 3) based on limited evidence of carcinogenicity in animals and inadequate evidence in humans. Although an excess of bladder cancer has been observed in aniline-dye workers, these workers were also exposed to other bladder carcinogens. Studies of aniline-dye workers not exposed to other carcinogens found no increased risk of cancer but had methodological problems that limited their interpretation.

**Studies of Cancer in Experimental Animals**

In carcinogenicity studies conducted in the late 1940s, dermal exposure to DAAB resulted in skin and lung tumors in some mice. DAAB is metabolized to benzene and aniline, both of which are carcinogenic in laboratory animals. Oral exposure to benzene induced multiple tumors at multiple sites in rats (Zymbal gland, oral cavity, and skin) and mice (lymphoma, Zymbal gland, lung, Harderian gland, and preputial gland) of both sexes. In addition, Tg.AC transgenic mice, which carry a v-Ha-ras oncogene, developed skin tumors and leukemia following dermal exposure to benzene. Rats exposed to aniline in the diet developed sarcoma of the spleen and other body organs. Therefore, DAAB has been concluded to be carcinogenic in animals, based on its metabolism to benzene and aniline.

**Genotoxicity**

DAAB is mutagenic in bacteria with metabolic activation, and it induces chromosomal aberrations in plants and micronuclei in the bone marrow cells of mice exposed *in vivo*. Benzene, a major metabolite of DAAB, has a pattern of genotoxicity consistent with patterns observed for other leukemia-inducing chemicals; it is effective in damaging chromosomes but relatively ineffective in inducing point mutations. Mutagenicity studies in bacteria have given mixed results without metabolic activation and generally negative results with metabolic activation. In mammalian in *vitro* assays, benzene induced chromosomal recombination and DNA adducts in human tissue, and it induced DNA phosphorylation and sister chromatid exchange and inhibited RNA synthesis in animal cells. In *in vivo* studies, benzene induced chromosomal aberrations, DNA damage, and micronuclei in mice and rats. Chromosomal aberrations have been detected in the bone morrow and lymphocytes of humans exposed occupationally to benzene; the most common damage observed is chromosomal breakage, but deletions and other alterations also have been reported. Aniline does not induce mutations in bacteria. In mammalian *in vitro* assays, aniline induced sister chromatid exchange and chromosomal aberrations, but not DNA damage. In *in vivo* animal studies, aniline induced sister chromatid exchange and micronuclei.

**Other Relevant Data**

*Absorption, distribution, and metabolism.* In disposition studies in rats and mice, DAAB was almost completely absorbed from the gastrointestinal tract but sparingly absorbed from skin. Following administration by each route, the absorbed portion of the dose was rapidly metabolized and excreted primarily in urine. Metabolites identified in blood (of rats) or urine (of rats and mice) were benzene, aniline, and their metabolites. Benzene was exhaled by rats and mice after oral administration of DAAB. The metabolic

conversion of DAAB to metabolites of benzene is consistent with the metabolic pathway for benzene, in which formation of benzene oxide is the initial step. Metabolites of DAAB in the blood of rats and the urine of rats and mice included hydroquinone, muconic acid, and phenylmercapturic acid, which share benzene oxide as a common intermediate. Studies with human liver slices also demonstrated that DAAB could be cleaved to yield metabolites of benzene and aniline.

The proposed metabolic pathway for DAAB is that it is cleaved reductively by liver enzymes or gut flora to form aniline, benzene, and nitrogen. Benzene and aniline are subsequently metabolized by cytochrome P-450 and conjugating enzymes. DAAB metabolism also results in the formation of a reactive phenyl radical, which could account for an additional risk of toxicity or carcinogenicity. Evidence for the possible involvement of the phenyl radical in DAAB mutagenicity may be seen in the fact that DAAB is mutagenic in *Salmonella typhimurium* with metabolic activation, whereas benzene and aniline are not.

*Toxicity studies*. Symptoms observed in animals exposed to DAAB (dermally, but without protection of the application site, to allow for oral exposure via grooming) were similar to those that would be anticipated for animals exposed to benzene and/or aniline and included decreased thymus weights (rats and mice) and increases in the weights of the heart (rats and mice), spleen (rats), liver (rats) and kidney (male rats and female mice). DAAB induced hematologic effects in rats and mice, including chemical-related methemoglobinemia and Heinz-body formation. DAAB-related non-neoplastic lesions in both rats and mice included hyperplasia and inflammation of the skin, lymphoid atrophy of the thymus, atrophy of the lymph nodes, and hematopoietic cell proliferation in the spleen. Non-neoplastic lesions in the heart, kidney, and liver also were observed in mice. The erythrocytes, thymus, and lymphoid system are major targets of benzene, whereas symptoms of aniline exposure include methemoglobin formation, anemia, increased spleen weight, and regenerative hematopoiesis. DAAB also appeared to induce toxicity not observed for aniline or benzene, including skin lesions at the application site.

*Potential mechanism of carcinogenicity.* Studies have demonstrated that in rats and mice, DAAB is quantitatively metabolized to benzene, a known human and animal carcinogen, and to the rat carcinogen, aniline. Symptoms observed in animals administered DAAB in short-term studies are similar to those anticipated for animals exposed to benzene and/or aniline. DAAB is mutagenic in *S. typhimurium* and induces micronuclei and chromosomal aberrations in the bone marrow of rodents. Like DAAB, benzene and aniline induce micronuclei in mice; however, DAAB induced more micronuclei in mice than did equimolar doses of benzene or a mixture of benzene and aniline, an effect suggested to be due to the formation of the phenyl radical during metabolism of DAAB.

## Table of Contents

**List of Tables**

## List of Figures

# 1 Introduction

Diazoaminobenzene (DAAB) is a triazene that is used as an intermediate in chemical synthesis, as a complexing agent, and as a polymer additive. It has been used as a propellant for the molding of rubbers and plastics and as a coupler to promote adhesion of natural rubber to steel tire cords (Kirk-Othmer 1982). Occupational exposure to DAAB occurs from its use as an intermediate during organic synthesis and in the manufacture of dyes and insecticides (Lewis 1997). Other exposures to DAAB may occur through its presence in cosmetics and food products. It has been identified as a contaminant in several dyes that have been permitted for use in ingested and externally applied drugs and cosmetics (Bailey 1985, Palmer and Mathews 1986).

DAAB was nominated by the National Institute of Environmental Health Sciences (NIEHS) for possible listing in the Report on Carcinogens based on the results of National Toxicology Program (NTP) toxicity studies which concluded that DAAB is metabolized to the known human carcinogen benzene (see NTP 2002b, IARC 1982a, 1987a) and showed significant increases in micronuclei in the bone marrow of mice exposed to DAAB. DAAB is also metabolized to aniline that has been shown to be carcinogenic in rats. In addition, some toxic effects associated with benzene (atrophy of the lymphoid tissue) and aniline (methemoglobinemia and Heinz-body anemia) were identified in the toxicity studies of DAAB, which lends additional support to the prediction that DAAB has similar toxic effects to benzene and aniline. This Background Document is based in large part on the NTP (2002a) Report on the Metabolism, Toxicity, and Predicted Carcinogenicity of Diazoaminobenzene.

## 1.1 Chemical identification

Diazoaminobenzene ($C_{12}H_{11}N_3$, mol wt 197.24, CASRN 136-35-6) also is known as 1,3-diphenyl-1-triazene, DPT, anilinoazobenzene, benzeneazoanilide, benzeneazoaniline, DAAB, alpha-diazoamidobenzol, 1,3-diphenyltriazine, *N*-(phenylazo)aniline, 1,3-diphenyl-triazene, diazobenzeneanilide, and *p*-diazoaminobenzene (HSDB 2001a, NTP 2002a). [The validity of the reported synonym *p*-diazoaminobenzene is uncertain, because DAAB does not contain para bonds.] The RTECS number for DAAB is XY265000. The structure of DAAB is illustrated in Figure 1-1.

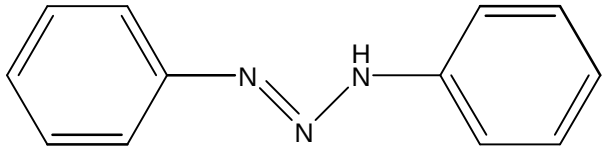



**Figure 1-1. Chemical structure of DAAB**

## 1.2 Physical-chemical properties

DAAB exists as small, golden-yellow crystals or an orange solid at room temperature. It melts at 98°C, decomposes at 130°C, and explodes at its boiling point of 150°C.

Decomposition products of DAAB include benzene, *o*- and *p*-aminodiphenyl, diphenylamine, and azobenzene. DAAB is soluble in ethyl alcohol, ethyl ether, benzene, pyridine, and hexane; it is insoluble in water. The physical and chemical properties of DAAB are summarized in Table 1-1.

**Table 1-1. Physical and chemical properties of DAAB**

| Property | Information | References |
|---|---|---|
| Molecular weight | 197.24 | Budavari *et al.* 1996 |
| Color | golden-yellow<br>orange | Budavari *et al.* 1996<br>NTP 2002a |
| Physical state | crystals<br>solid | Budavari *et al.* 1996<br>NTP 2002a |
| Melting point (°C) | 98 | Budavari *et al.* 1996 |
| Boiling point (°C) | 150 | NTP 2002a |
| Vapor density | 6.8 | Lewis 2000 |
| Solubility | | |
| water at 22°C | < 1 mg/mL | NTP 2002a |
| acetone at 18°C | 100 mg/mL | NTP 2001 |
| benzene | soluble | NTP 2001 |
| 95% ethanol at 18°C | 10–50 mg/mL | NTP 2001 |
| ether | soluble | NTP 2001 |
| Decomposition products | benzene, *o*- and *p*-aminodiphenyl, diphenylamine, and azobenzene | Mortimore *et al.* 1979 |

## 1.3 Identification of metabolites

DAAB is metabolized to benzene and aniline and to their metabolites. See Section 6.1.2 for further discussion of the metabolites of DAAB.

# 2 Human Exposure

## 2.1 Use

DAAB is used as a chemical intermediate, complexing agent, and polymer additive (Mathews and De Costa 1999). It also has uses in organic synthesis and manufacture of dyes and insecticides (Lewis 1997) and as a dopant for laser ablation of polymethylmethacrylate (Bolle *et al.* 1990). As a polymer additive, DAAB has been listed in a European patent as a chemical that may be used as a coupler to promote adhesion of natural rubber to steel tire cords (Shemenski and Starinshak 1982). It also is listed in a U.S. patent as a chemical that may be used as a blowing agent in the production of a foamed polymeric material having a camouflage appearance (Raad 1993).

DAAB has been identified as a contaminant in D&C Red No. 33, FD&C Yellow No. 5, and FD&C Yellow No. 6; all are permitted for use in drugs and cosmetics, and the latter two are permitted in food (FDA 2001, 21 CFR 74). D&C Red No. 33 is a color additive permitted for use in ingested and externally applied drugs, lipsticks, and cosmetics (Bailey 1985). Bailey (1985) identified DAAB in 9 of 11 samples of commercial D&C Red No. 33 at concentrations of up to 439 ppb, with an average of 99 ppb. DAAB also was identified at concentrations of 68 and 110 ppb in two "pharmacology samples" from the U.S. Food and Drug Administration's (FDA's) animal feeding studies to evaluate the toxicity of D&C Red No. 33. The common name for FD&C Yellow No. 5 is tartrazine, and this colorant is a common constituent of cosmetics, such as shampoos, hand and body lotions, bath and shower gels, facial cleansers, and sunless tanning products; medications, including pediatric antihistamines; and foods and beverages, including candy, baked goods, ice cream, and cereal. The FDA's risk evaluation of FD&C Yellow No. 5 concluded that the normal use of this colorant would not result in significant exposure to carcinogenic impurities, including DAAB (Palmer and Mathews 1986). FD&C Yellow No. 6 is commonly known as sunset yellow and is used in beverages, baked goods, desserts, and ice cream (Fraser 2002).

## 2.2 Production

DAAB may be produced by the rapid reaction of aniline with isoamyl nitrate, with a product yield of 67% (Smith and Ho 1990), or by diazotization of aniline dissolved in hydrochloric acid with sodium nitrite, followed by addition of a concentrated solution of sodium acetate (HSDB 2001). It also is formed as an intermediate during the preparation of iodobenzene from aniline and through the interaction of nitrous acid and an alcoholic solution of aniline (NTP 2002a). In a patented process, DAAB may be formed by the reaction of aniline with nitrogen oxides, which are produced via the oxidation of ammonia (Detrick 1977, Herkes 1977).

No data were found on U.S. production of DAAB. Chem Sources (2001) identified seven U.S. suppliers of DAAB. No import data for DAAB as an individual compound were available. However, U.S. imports of DAAB and *p*-aminoazobenzenedisulfonic acid combined totaled 94,237 lb (42,835 kg) from January through October 2001 (U.S. ITA 2001).

Although production data for DAAB could not be found in current literature, production data for dyes that contain DAAB as an impurity (D&C Red No. 33, FD&C Yellow No. 5, and FD&C Yellow No. 6) were found. The United States International Trade Commission (USITC) reported production of these dyes from 1980 to 1994, as summarized in Table 2-1.

**Table 2-1. U.S. production of dyes that contain DAAB as an impurity (in pounds)**

| | Year[a] | | | | | |
|---|---|---|---|---|---|---|
| **Dye** | **1980** | **1986** | **1987** | **1992** | **1993** | **1994** |
| D&C Red No. 33 | –[b] | 5,000 | 7,000 | 12,000 | NR | 10,000 |
| FD&C Yellow No. 5 | 1,527,000 | 1,569,000 | 1,618,000 | NR | NR | NR |
| FD&C Yellow No. 6 | 1,206,000 | 1,528,000 | 1,153,000 | NR | 1,307,000 | NR |

Sources: USITC 1980, 1987, 1988, 1990, 1991, 1993, 1994a, 1994b, 1995.

[a]For the years 1989–1991, all three dyes reportedly were produced; however, specific production data were not provided for any of the three. NR = Production values not reported because of confidentiality issues.

[b]Although production of this dye was reported, specific production data were not provided.

The FDA regulates color additives for use in food, drugs, cosmetics, and medical devices in the United States. The FDA lists all approved color additives and requires domestic and foreign manufacturers of these colors to submit samples from each batch of color produced. FDA scientists test these samples to confirm that each batch is within established specifications that restrict the levels of impurities allowed in the color (FDA 2001). Table 2-2 summarizes total amounts of D&C Red No. 33, FD&C Yellow No. 5, and FD&C Yellow No. 6 certified by the FDA for 1997 through 2001.

## 2.3 Analysis

DAAB has been identified as a contaminant in 1-aryl-3,3-dialkyl triazine compounds by thin-layer chromatography. Solvent extraction and reversed-phase high-performance liquid chromatography (HPLC) were used to analyze for the compound in D&C Red No. 33 (Bailey 1985).

## 2.4 Environmental occurrence

No information was found regarding environmental occurrence of DAAB.

## 2.5 Environmental fate

No information was found regarding the environmental fate of DAAB.

**Table 2-2. Total certification of color additives (in pounds)**

| Dye[b] | Year | | | | |
|---|---|---|---|---|---|
| | 1997 | 1998 | 1999 | 2000 | 2001 |
| D&C Red No. 33 | | | | | |
| Primary | 24,568 | 21,027 | 30,661 | 42,928 | 25,940 |
| Repacks | –[a] | – | – | 1,513 | 15,689 |
| Lakes | 22,125 | 9,212 | 8,531 | 45,960 | 18,367 |
| FD&C Yellow No. 5 | | | | | |
| Primary | 2,086,314 | 2,288,198 | 2,597,407 | 2,202,549 | 2,733,240 |
| Repacks | – | – | – | 3,455 | 8,262 |
| Lakes | 1,223,794 | 1,403,629 | 1,191,999 | 1,043,291 | 1,451,570 |
| FD&C Yellow No. 6 | | | | | |
| Primary | 1,934,898 | 2,173,554 | 2,494,268 | 2,275,356 | 2,316,832 |
| Repacks | – | – | – | – | 27,784 |
| Lakes | 787,084 | 977,373 | 975,228 | 812,453 | 1,199,015 |

Source: FDA 2001.

[a]– = information not provided.

[b]Primary is defined as the color additive itself and is water soluble. Repacks refer to the packaging for a second time of a previously certified color additive. Lakes are the dye form attached to an aluminum or calcium substrate to make it insoluble.

## 2.6 Environmental exposure

The presence of DAAB as an impurity in dyes and colorants, such as FD&C Yellow No. 5, could result in very low-level consumer exposure by the oral and dermal routes. In 1977, the National Academy of Sciences surveyed the amount of certified FD&C colorants consumed by the U.S. population. For 12,000 persons over two years of age surveyed for 14 days, the results showed average daily intakes of 43 mg for FD&C Yellow No. 5 and 37 mg for FD&C Yellow No. 6 (Feingold 2002). [Although no data are available for exposure levels of DAAB in dyes and colorants, theoretical maximum daily exposures of 1.7 ng for FD&C Yellow No. 5 and 1.5 ng for FD&C Yellow No. 6 may be calculated, based on the average daily intakes calculated by Feingold (2002) and the maximum allowable levels of DAAB in colorants under the FDA regulations (see Table 2-3).]

## 2.7 Occupational exposure

Occupational exposure to DAAB could occur from its use as a chemical intermediate and polymer additive. DAAB is explosive and is harmful to the respiratory tract, skin, and eyes through inhalation or dermal contact (Aldrich Chemical 2002). DAAB was not listed in the National Occupational Exposure Survey conducted by the National Institute for Occupational Safety and Health between 1981 and 1983 (NTP 2002a).

## 2.8 Biological indices of exposure

No information was found regarding biological indices of exposure to DAAB.

## 2.9 Regulations

No DAAB-specific regulations were found. The FDA regulates FD&C Yellow No. 5 and FD&C Yellow No. 6 for use as color additives in foods, drugs, and cosmetics and D&C Red No. 33 for use as a color additive in drugs and cosmetics. Special labeling is required for drugs that contain FD&C Yellow No. 5 and FD&C Yellow No. 6. No regulations from other governmental agencies were located for D&C Red No. 33, FD&C Yellow No. 5, or FD&C Yellow No. 6. Table 2-3 summarizes the FDA regulations for these three dyes.

**Table 2-3. FDA regulations**

| Regulatory action | Effect of regulation or other comments |
|---|---|
| 21 CFR 74 – PART 74 – LISTING OF COLOR ADDITIVES SUBJECT TO CERTIFICATION. Promulgated: 42 FR 15654, 3/22/77. U.S. Codes: 21 U.S.C. 321, 341, 342, 343, 348, 351, 352, 355, 361, 362, 371, and 379e. | Lists color additives that are subject to certification in drugs, cosmetics, and medical devices. FD&C Yellow No. 5 and FD&C Yellow No. 6 may be safely used for coloring food, and D&C Red No. 33 may be safely used for coloring ingested drugs, other than mouthwashes and dentrifices, in amounts not to exceed 0.75 mg per daily dose of the drug. The maximum level of DAAB impurity in FD&C Yellow No. 5 is 40 ppb. The maximum level of DAAB impurity in FD&C Yellow No. 6 is 40 ppb. The maximum level of DAAB impurity in D&C Red No. 33 is 125 ppb. |
| 21 CFR 82 – PART 82 – LISTING OF CERTIFIED PROVISIONALLY LISTED COLORS AND SPECIFICATIONS. Promulgated: FD&C Yellow No. 5: 51 FR 24519, 7/7/86; FD&C Yellow No. 6: 52 FR 21509, 6/8/87; D&C Red No. 33: 53 FR 33121, 8/30/88. U.S. Codes: 21 U.S.C. 371, 379e, and 379e note | General specifications are given for colors and certifiable mixtures that can be used in foods, drugs, and cosmetics. FD&C Yellow No. 5 and FD&C Yellow No. 6 are color additives that are allowed to be used in foods, drugs, and cosmetics. D&C Red No. 33 may be used in drugs and cosmetics. |
| 21 CFR 201 – PART 201 – LABELING. Promulgated: 40 FR 13998, 3/27/75. U.S. Codes: 21 U.S.C. 321, 331, 351, 352, 353, 355, 358, 360, 371, 374, and 379e; 42 U.S.C. 216, 241, 262, and 264. | There must be labels for over-the-counter and prescription drugs containing FD&C Yellow No. 5 and FD&C Yellow No. 6 as color additives. |

Source: The regulations in this table have been updated through the 2001 Code of Federal Regulations 21 CFR, December 31, 2001.

# 3 Human Cancer Studies

No human studies were identified in which exposure to DAAB is specifically mentioned. Because nomination of DAAB was based on its metabolism to benzene and aniline, the human cancer effects of these metabolites are summarized briefly in this section.

## 3.1 Benzene

Benzene is listed in the Report on Carcinogens as a *known to be human carcinogen* based on sufficient evidence of carcinogenicity in humans (NTP 2002b). The International Agency for Research on Cancer (IARC) also classified benzene as carcinogenic to humans (Group 1) based on sufficient evidence in humans (IARC 1982a, 1987a). The RoC and IARC based their conclusion on case reports, case series, case-control studies, and cohort studies that reported an association between exposure to benzene and leukemia (primarily acute myeloid leukemia), with the strongest evidence coming from the cohort studies.

The International Programme on Chemical Safety (IPCS) reviewed health effects of benzene in 1993. This report included a review of human epidemiological studies of exposure to benzene and cancer, including studies reviewed by IARC (1987a) and studies published since the 1987 IARC review. Increased risk of leukemia was identified in studies of benzene-exposed shoemakers, chemical and rubber workers, and workers in oil refineries and a study of benzene workers in various locations, with standardized mortality ratios (SMRs) ranging from 1.6 to 5.7. The IPCS task force concluded that the most consistent evidence was found for an association between benzene exposure and myeloid leukemia, although other types of leukemia, lymphoma, and multiple myeloma also were reported. Increased risks of other types of cancer have been reported in some studies, but the findings are not consistent. Some studies reported an exposure-response relationship between benzene exposure and leukemia, with very high SMR values (between 50 and 60 in two studies) for the groups with highest exposure.

Since the IARC review, Savitz and Andrews (1997) reviewed 18 community-based and 16 industry-based studies that provided data on benzene exposure and lymphatic and hematopoietic cancer. Their purpose was to look at the relationship between benzene exposure and types of leukemia other than acute myeloid leukemia (which is accepted to be associated with benzene exposure) and other types of hematopoietic cancers. Most studies found an association between benzene exposure and total lymphatic and hematopoietic cancer, total leukemia, and specific histologic types of leukemia, including chronic lymphocytic leukemia, as well as acute myeloid leukemia. Little evidence was found for an association between benzene exposure and multiple myeloma or non-Hodgkin's lymphoma. The authors concluded that the evidence supported a general association of benzene exposure with leukemia, rather than a specific association with acute myeloid leukemia.

## 3.2 Aniline

IARC classified aniline as not classifiable as to its carcinogenicity (Group 3), based on limited evidence in animals and inadequate evidence in humans (IARC 1982b, 1987b). IARC noted that although an excess of bladder cancer had been observed in aniline-dye workers, studies of workers exposed to aniline but not to other known bladder carcinogens had not reported an increased risk of cancer. However, these studies had methodological problems due to incomplete follow-up of workers who left the industry and to the absence of estimates of expected numbers of bladder cancer cases (IARC 1982b, 1987b).

# 4 Studies of Cancer in Experimental Animals

Kirby (1947, 1948) conducted carcinogenicity studies in mice using a chemical identified as *p*-diazoaminobenzene and DAAB. The chemical was purified by solution in ether or benzene, poured through a tower of alumina, concentrated, and precipitated by addition of petroleum ether. The purified chemical was described as a light tan powder with a melting point of 98°C. There is some question regarding the identity of this compound, because DAAB does not have para bonds and is golden-yellow to orange in color (see Section 1.2). Nevertheless, *p*-diazoaminobenzene is listed as a synonym for DAAB in some sources (see Section 1), and the melting point is correct. Furthermore, Kirby (1947) noted that DAAB is readily rearranged by chemical means to yield *p*-aminoazobenzene. This fact appears to establish the study chemical as DAAB, because chemical rearrangement of DAAB to *p*-aminoazobenzene is part of an industrial process for making phenylenediamine (Kirk-Othmer 1996). Although experimental details of these studies were lacking and the number of animals used was small, the data from these studies are presented in Section 4.1. No other carcinogenicity studies were identified.

Because DAAB is metabolized to benzene and aniline, the experimental carcinogenicity data for these chemicals reported by the National Cancer Institute (NCI 1978), the National Toxicology Program (NTP 1986), and the Chemical Industry Institute of Toxicology (CIIT 1982) are summarized in Section 4.2. Section 4.3 presents recent data on the carcinogenicity of benzene administered to genetically altered mice.

## 4.1 DAAB

Kirby (1947) investigated the effects of DAAB exposure by the oral, subcutaneous (s.c.) injection, and dermal routes. No control groups were reported for any of the studies.

For oral exposure, 6 male and 6 female mice (strain not reported) were fed a diet of rat cake powder containing 50 mg of DAAB per 100 g of diet; 10 additional mice were fed cake powder with 100 mg of DAAB per 100 g of diet; and 7 male and 3 female mice were fed a special restricted diet containing 100 mg of DAAB per 100 g of diet. Two male and all the female mice in the group given DAAB at 50 mg/100 g of cake powder died within 39 days as a result of liver and kidney toxicity. The remaining 4 male mice survived 122 to 125 days. Three mice in the group given DAAB at 100 mg/100 g of cake powder survived 240 to 291 days, and the other 7 mice died or were sacrificed on day 29. In the third group, 4 males and all 3 females died within 64 days. Two of the remaining males died by day 95, and one survived 331 days. No stomach lesions or tumors were reported in any of the three exposure groups.

In the injection experiments, 10 male and 10 female mice were given an initial s.c. injection in the right flank of 0.25 mL of arachis oil containing 2 mg of DAAB. About one month after the first injection, the survivors (1 male died) were injected with 4 mg of DAAB in the left flank. After 190 days, the surviving mice (8 males and 10 females) received a third injection of 10 mg of DAAB in the right flank; 4 males and 7 females died within a week of the last injection. Two mice of each sex survived 323 to 329 days. No tumors were reported in any of the mice.

The dermal studies consisted of painting mice (number not reported) in the interscapular region with a 0.5% solution of DAAB in acetone; after unspecified periods, the concentration was increased to 1%, then 2%, and finally 5%. Eight mice survived to be painted with the 5% solution, two of which developed squamous carcinoma. These mice survived for 346 to 601 days.

Kirby (1948) duplicated the dermal exposure study described above using a 5% solution of DAAB and added another experiment where DAAB was dissolved in a 0.5% solution of croton oil in acetone. Applications were made to the nape of the shaved neck, three times per week for up to 545 days. Croton oil had no effect on the outcome. Of 17 mice that survived more than 400 days, 5 developed squamous papilloma and 5 others developed squamous carcinoma at the application site. Two mice developed pulmonary adenoma, and one mouse developed pulmonary adenocarcinoma.

### 4.2 Benzene and aniline

The NTP (1986) conducted a two-year carcinogenicity assay of benzene in F344/N rats and $B6C3F_1$ mice. The NCI (1978) investigated the carcinogenicity of aniline hydrochloride in F344/N rats and $B6C3F_1$ mice, and CIIT (1982) investigated the carcinogenicity of aniline hydrochloride in CD-F rats. Table 4-1 shows the lowest doses associated with increased incidences of tumors of various types observed in these studies. In most cases, the lowest dose tested (25 mg/kg body weight [b.w.] in mice, 50 mg/kg b.w. in rats) induced increased incidences of at least one type of tumor. Exposure-response data from these studies are summarized in Tables 4-2a and 4-2b (benzene) and Table 4-3 (aniline). The data in Tables 4-1, 4-2a, 4-2b, and 4-3 all were derived from the NCI (1978), NTP (1986), and CIIT (1982) reports.

In the benzene studies, survival was significantly decreased in high-dose male rats, mid- and high-dose female rats, and in high-dose male and female mice; therefore, tumor incidence data in the exposed groups were adjusted for intercurrent mortality. Benzene induced Zymbal gland, oral cavity, and skin tumors in rats and Zymbal gland, lung, Harderian gland, ovarian, and preputial gland tumors and malignant lymphoma in mice. Aniline induced splenic sarcomas in rats but was not carcinogenic in mice.

**Table 4-1. Lowest doses of benzene or aniline causing cancer in rats and mice**

| Chemical and strain | Exposure route[a] | Exposure duration (wk) | Dose level[b] (mg/kg b.w.) | Tumor types |
|---|---|---|---|---|
| Benzene | | | | |
| Rat, F344/N | | | | |
| Male | gavage | 103 | 50[c] | oral cavity and skin squamous-cell papilloma or carcinoma |
| | | | 100 | Zymbal gland carcinoma |
| Female | gavage | 103 | 25[c] | Zymbal gland carcinoma |
| | | | 50 | oral cavity squamous-cell papilloma or carcinoma |
| Mouse, $B6C3F_1$ | | | | |
| Male | gavage | 103 | 25[c] | Harderian gland adenoma |
| | | | 50 | Zymbal gland and preputial gland carcinoma, alveolar/bronchiolar adenoma or carcinoma, malignant lymphoma |
| Female | gavage | 103 | 25[c] | malignant lymphoma |
| | | | 50 | alveolar/bronchiolar adenoma or carcinoma, ovarian granulosa-cell tumor, mammary gland carcinoma |
| | | | 100 | Harderian gland and Zymbal gland carcinoma |
| Aniline | | | | |
| Rat, CD-F | | | | |
| Male | feed | 104 | 100 | splenic sarcoma |
| Rat, F344/N | | | | |
| Male | feed | 104 | 130[c,d] | splenic hemangiosarcoma |
| | | | 260[d] | splenic and body-cavity sarcoma |
| Female | feed | 104 | 240[d,e] | splenic and body-cavity sarcoma |

Sources: NCI 1978, NTP 1986, CIIT 1982.

[a]Gavage exposure was for 5 days per week.

[b]The lowest dose at which tumor incidence exceeded control values.

[c]The lowest dose tested.

[d]The average daily dose (mg/kg per day) is approximate, assuming an average daily feed consumption of 11 g (females) and 15 g (males), an average body weight of 270 g (females) and 350 g (males), and an average concentration of aniline in the feed of 3,000 or 6,000 ppm.

[e]Based on the total incidence (7/50) of fibrosarcoma or sarcoma (spleen and body cavity combined), compared with historical controls. Results were not significantly different compared to concurrent controls.

**Table 4-2a. Tumor incidence in F344 rats exposed to benzene by gavage**

| Sex | Dose (mg/kg b.w.) | Tumor incidence[a] (%) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Zymbal gland carcinoma | Oral cavity (squamous cell) | | | Skin (squamous cell) | | |
| | | | Papilloma | Carcinoma | Combined | Papilloma | Carcinoma | All |
| M | 0 | 2/32 (7) | 1/50 (2) | 0/50 (0) | 1/50 (2) | 0/50 (0) | 0/50 (0) | 1/50 (3) |
| | 50 | 6/46 (15) | 6/50 (18) | 3/50 (7) | 9/50 (24)* | 2/50 (7) | 5/50 (15)* | 7/50 (22)* |
| | 100 | 10/42 (29)* | 11/50 (37)*** | 5/50 (15)* | 16/50 (49)*** | 1/50 (4) | 3/50 (10) | 5/50 (17) |
| | 200 | 17/42 (56)*** | 13/50 (48)*** | 7/50 (33)*** | 19/50 (69)*** | 5/50 (27)** | 8/50 (30)*** | 11/50 (45)*** |
| F | 0 | 0/45 (0) | 1/50 (2) | 0/50 (0) | 1/50 (2) | 1/50 (NR) | 0/50 (0) | 1/50 (NR) |
| | 25 | 5/40 (13)* | 4/50 (11) | 1/50 (3) | 5/50 (13) | 0/50 (0) | 0/50 (0) | 0/50 (0) |
| | 50 | 5/44 (14)* | 8/50 (21)** | 4/50 (10)* | 12/50 (29)*** | 1/50 (NR) | 0/50 (0) | 1/50 (NR) |
| | 100 | 14/46 (42)*** | 5/50 (16)* | 5/50 (15)** | 9/50 (28)*** | 0/50 (0) | 1/50 (NR) | 1/50 (NR) |

Source: NTP 1986.

*$P < 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ compared with controls (life-table tests).

[a]Number of animals with tumors/number of animals at risk. Percentages are based on the Kaplan-Meier estimated incidences after adjusting for intercurrent mortality. NR = not reported.

## Table 4-2b. Tumor incidence and dose-response data in B6C3F$_1$ mice exposed to benzene by gavage

| Sex | Dose (mg/kg b.w.) | Tumor incidence (%)[a] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Zymbal gland carcinoma | Malignant lymphoma | Lung (alveolar/bronchiolar) | | | Harderian gland combined | Preputial gland carcinoma[b] | Ovary granulosa cell[c] | Mammary gland carcinoma[d] |
| | | | | Adenoma | Carcinoma | Combined | | | | |
| M | 0 | 0/43 (0) | 4/49 (12) | 6/49 (19) | 5/49 (16) | 10/49 (30) | 1/49 (4) | 0/21 (0) | NAP | NAP |
| | 25 | 1/34 (3) | 9/48 (31) | 6/48 (21) | 11/48 (36) | 16/48 (49) | 10/46 (36)** | 5/28 (22) | | |
| | 50 | 4/40 (29)* | 9/50 (42)* | 8/50 (29) | 12/50 (42)* | 19/50 (60)** | 13/49 (52)*** | 19/29 (82)*** | | |
| | 100 | 21/39 (88)*** | 15/49 (69)*** | 12/49 (42)** | 14/49 (59)*** | 21/49 (71)*** | 14/48 (61)*** | 31/35 (96)*** | | |
| F | 0 | 0/43 (0) | 15/49 (42) | 4/49 (13) | 0/49 (0) | 4/49 (13) | 5/48 (17) | NAP | 1/47 (3) | 0/49 (0) |
| | 25 | 0/32 (0) | 24/45 (68)* | 2/42 (7) | 3/42 (13) | 5/42 (19) | 6/44 (24) | | 1/44 (4) | 2/45 (7) |
| | 50 | 1/37 (5) | 24/50 (63)* | 5/50 (16) | 6/50 (23)** | 10/50 (33)* | 10/50 (27) | | 6/49 (20)* | 5/50 (16)* |
| | 100 | 3/31 (19)* | 20/49 (54)* | 9/49 (44)* | 6/49 (27)** | 13/49 (57)*** | 10/47 (41)* | | 7/48 (29)** | 10/49 (33)*** |

Source: NTP 1986.

*$P < 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ compared with controls (life-table tests).

[a]Number of animals with tumors/number of animals at risk. Percentages are based on the Kaplan-Meier estimated incidences after adjusting for intercurrent mortality. NAP = not applicable.

[b]All types.

[c]Increased incidences of benign mixed tumors at the mid and high dose also were reported (0/47, 1/44, 12/49, 7/48).

[d]Increased incidence of carcinosarcoma at the high dose also were reported (0/49, 0/45, 1/50, 4/49).

**Table 4-3. Tumor incidence in rats exposed to aniline hydrochloride in the diet**

| Strain | Sex | Conc. in diet or daily dose[a] | No. with tumors/no. examined (%) | | | Reference |
|---|---|---|---|---|---|---|
| | | | Fibrosarcoma or sarcoma NOS[b] (spleen) | Hemangio-sarcoma (spleen) | Combined sarcomas (multiple body cavity organs)[c] | |
| F344 | male | 0 | 0/25 (0) | 0/25 (0) | 0/25 (0) | NCI 1978 |
| | | 3,000 ppm | 7/50 (14) | 19/50 (38)*** | 2/50 (4) | |
| | | 6,000 ppm | 9/46 (20)* | 20/46 (43)*** | 9/48 (19)* | |
| F344 | female | 0 | 0/23 (0) | 0/23 (0) | 0/24 (0) | NCI 1978 |
| | | 3,000 ppm | 0/50 (0) | 1/50 (2) | 1/50 (2) | |
| | | 6,000 ppm | 3/50 (6) | 2/50 (4) | 4/50 (8) | |
| CD-F | male | 0 | 0/123 (0) | 0/123 (0) | NR | CIIT 1982 |
| | | 10 mg/kg | 0/129 (0) | 0/129 (0) | NR | |
| | | 30 mg/kg | 1/128 (1)[d] | 0/128 (0) | NR | |
| | | 100 mg/kg | 28/130 (22)[e,f] | 6/130 (5)[f] | NR | |
| CD-F | female | 0 | 0/129 (0) | 0/129 (0) | NR | CIIT 1982 |
| | | 10 mg/kg | 0/129 (0) | 0/129 (0) | NR | |
| | | 30 mg/kg | 0/130 (0) | 0/130 (0) | NR | |
| | | 100 mg/kg | 0/130 (0) | 1/130 (1) | NR | |

*$P < 0.05$, ***$P \leq 0.001$ compared with controls.

[a]Concentrations in the diet in the CIIT study were varied throughout the study: low dose = 117–248 ppm, mid dose = 310–762 ppm, high dose = 1,129–2,360 ppm.

[b]NOS = not otherwise specified.

[c]NR = not reported.

[d]Neoplasm identified as a stromal sarcoma.

[e]Neoplasms included 3 fibrosarcomas, 21 stromal sarcomas, 1 capsular sarcoma, and 3 osteogenic sarcomas.

[f]*P*-values were not provided.

## 4.3 Benzene studies in genetically altered mice

### *4.3.1 Dermal studies in transgenic mice*

This section reviews three relatively recent studies (Blanchard *et al.* 1998, Spalding *et al.* 1999, French and Saulnier 2000) that examined the carcinogenicity of benzene applied to the skin of transgenic mice. The Tg.AC transgenic mouse model was used in each of these studies. These mice carry a v-Ha-*ras* oncogene, which imparts the characteristics of genetically initiated skin reported to be similar to those of the mouse-skin tumorigenesis model. The untreated skin of these animals appears normal, and spontaneous skin tumors are rare. However, exposure to a carcinogen activates the transgene, resulting in squamous-cell papilloma.

Blanchard *et al.* (1998) investigated the effects of dermal application of 200 µL of neat benzene, three times per week for 20 weeks, on tumor incidences in male and female homozygous and hemizygous Tg.AC mice. Benzene induced skin tumors in all groups of benzene-treated mice; however, tumor incidence was higher in the homozygous mice than the hemizygous mice (Table 4-4). Statistical comparisons with the control groups were not provided.

Spalding *et al.* (1999) applied benzene in doses of 200 (7,000 mg/kg), 400, or 800 µL, twice per week for 20 weeks. Acetone was used as a negative control. The mid and high doses were applied as two or four 200 µL portions of neat benzene. The low-dose was applied as a 1:1 solution of benzene in acetone (i.e. 100 µL of benzene in 100 µL of acetone). Chemicals were applied in the morning and afternoon. Groups of 15 to 20 female homozygous Tg.AC mice were used. Benzene induced papillomas in a dose-dependent manner (Table 4-4).

French and Saulnier (2000) applied benzene to Tg.AC mice in doses of 450 or 800 µL per week for 14 to 26 weeks. The low dose was applied three times per week as 150 µL benzene in 50 µL acetone, and the high dose was applied twice a week as two 200-µL portions of neat benzene. The authors reported dose-dependent increases in papilloma and spindle-cell tumors but did not provide the data. The incidence of granulocytic leukemia was significantly increased ($P < 0.05$) at both the low dose (4/14, 29%) and the high dose (11/15, 73%). None of the 19 vehicle-control animals developed leukemia.

**Table 4-4. Skin tumor incidence in Tg.AC mice dermally exposed to benzene**

| Genotype | Sex | Exposure level (µL/wk) | Tumor incidence[a] (%) | Tumor multiplicity (tumors/mouse ± SD) | Reference |
|---|---|---|---|---|---|
| Hemizygous | M | 0 | 6/65 (9) | NR[b] | Blanchard *et al.* 1998 |
| | M | 600 | 3/10 (30) | 16.0 ± 3.6 | |
| | F | 0 | 2/65 (3) | NR[c] | |
| | F | 600 | 4/10 (40) | 17.3 ± 2.8 | |
| Homozygous | M | 600 | 10/10 (100) | 15.9 ± 4.7 | Blanchard *et al.* 1998 |
| | F | 600 | 9/10 (90) | 15.6 ± 5.0 | |
| Homozygous | F | 0 | 3/5 (60)[d] | 1.4 ± 1.7 | Spalding *et al.* 1999 |
| | F | 400 | 7/10 (70) | 7.0 ± 10.3 | |
| | F | 800 | 8/10 (80) | 10.6 ± 8.5* | |
| | F | 1,600 | 10/10 (100)* | 12.6 ± 8.3* | |

*$P < 0.05$ compared with controls (the Blanchard *et al*. 1998 study did not provide statistical comparisons with controls).

[a]Number of animals with tumors/number of animals at risk.

[b]Not reported; 5 mice had 1 tumor, and 1 mouse had 4 tumors; however, 2 tumors were observed before exposure.

[c]Not reported; each mouse only had 1 tumor.

[d]Among all other vehicle-control groups, the incidence was 7/67 (10%). The high incidences in this group were wound induced.

### *4.3.2 Gavage and inhalation studies in* p*53 heterozygous mice*

In recent years the C57BL/6 *Trp*53 heterozygous (N5) mouse ($p53^{\pm}$ mice) has been used as an alternative model for carcinogenicity testing. Three inhalation studies (Boley *et al.* 2000, 2002, Healy *et al.* 2001) and two gavage studies (French *et al.* 2001, Hulla *et al.* 2001) of benzene exposure in these mice were reviewed.

Nearly 90% (24 of 27) of benzene-induced thymic lymphomas exhibited loss of the functional *p*53 allele locus, compared with 67% (4 of 6) in spontaneous tumors (Boley *et al.* 2000). Five different patterns of loss of heterozygosity (LOH) were observed in benzene-induced tumors, and six LOH patterns were observed in spontaneous tumors, with only one pattern common to both. Furthermore, 83% of these tumors retained two copies of the disrupted *p*53 locus, indicating that benzene exposure induced a high frequency of LOH on chromosome 11 in these mice which likely was mediated by aberrant chromosomal recombinations. Other inhalation studies with these mice indicated that benzene exposure (100 ppm [for 30 hours per week] or 200 ppm [for 15 hours per week] for up to six months) induced micronuclei in red blood cells and reticulocytes (Healy *et al.* 2001) and altered the mRNA expression of *p*53 target genes involved in cell-cycle control and apoptosis (Boley *et al.* 2002).

Exposure to benzene by gavage at a dose of 100 to 200 mg/kg b.w. per day for 26 weeks caused $p53^{\pm}$ mice to develop tumors with a high frequency of LOH (French *et al.* 2001, Hulla *et al.* 2001) (see Table 4-5). French *et al.* (2001) reported that benzene induced sarcomas around the head and neck or thoracic cavity and some thymic lymphomas. Hulla *et al.* (2001) also reported a high incidence of subcutaneous sarcomas, which were not observed in the NTP bioassay (see Section 4.2).

**Table 4-5. Loss of heterozygosity in tumors induced in heterozygous $p53^{\pm}$ mice exposed to benzene by gavage**

| Group | Daily dose (mg/kg b.w.) | Tumor[a] incidence (%) | LOH (%) | Reference |
|---|---|---|---|---|
| Control | 0 | 0/10 (0) | 0/10 (0) | French *et al.* 2001 |
| Exposed | 200 | 20/39 (51)** | 13/19 (68)*** | |
| Exposed | 100 | 19/39 (49)[b] | 13/16 (81)[b] | Hulla *et al.* 2001 |

$^{**}P < 0.01$, $^{***}P < 0.001$ compared with controls.

[a]Primarily subcutaneous sarcomas. French *et al.* (2001) reported 3 thymic lymphomas and 1 acinar carcinoma of the pancreas.

[b]Statistical analysis not reported.

## 4.4 Summary

In carcinogenicity studies conducted with DAAB in the late 1940s, dermal exposure resulted in skin and lung tumors in some mice. Furthermore, DAAB is metabolized to benzene and aniline, both of which are carcinogenic in laboratory animals. Oral exposure to benzene induced multiple tumors at multiple sites in rats and mice of both sexes. Rats

exposed to aniline in the diet developed sarcomas of the spleen and other body organs. In addition, transgenic (Tg.AC) mice developed skin tumors and leukemia following dermal exposure to benzene, and *p*53 heterozygous mice developed thymic lymphomas and various sarcomas following inhalation or gavage exposure to benzene. Therefore, DAAB is expected to be carcinogenic in animals, based on its metabolism to benzene and aniline.

# 5 Genotoxicity

Limited information is available on the genotoxicity of DAAB (see Section 5.3). However, there is a great deal of genotoxicity information available on benzene and some information on aniline, the principal metabolites of DAAB. This information is summarized in Sections 5.1 (benzene) and 5.2 (aniline).

## 5.1 Benzene

The genotoxicity of benzene has been investigated in a large number of *in vitro* and *in vivo* tests, and several recent reviews have been published (ATSDR 1997, Eastmond 2000, Whysner 2000). The following sections present a brief overview of genotoxic effects reported for benzene in *in vitro* and *in vivo* studies.

### *5.1.1* In vitro *assays*

The genotoxicity of benzene has been studied extensively in a variety of *in vitro* assays in prokaryotic and eukaryotic systems. *In vitro* studies have shown both positive and negative results with metabolic activation and generally negative results without metabolic activation for gene mutation in *Salmonella typhimurium* (at concentrations of 3 to 1,000 ppm) and *Escherichia coli* (10 µL). Positive results have been reported for DNA phosphorylation in rat liver epithelial cells, RNA synthesis inhibition in rat liver mitoplasts and in rabbit and cat bone marrow mitoplasts (1 mM), and DNA adduct formation in human cells (12.5 to 50 µg/mL) have been reported. In addition, benzene has been shown to cause sister chromatid exchange (SCE) in Chinese hamster ovary cells and intrachromosomal recombination in human lymphoblastoid cell culture (ATSDR 1997).

### *5.1.2* In vivo *assays*

Benzene has been shown to be genotoxic in *in vivo* studies in animals and humans (ATSDR 1997). Studies have shown that benzene is weakly effective in inducing point mutations and that it binds inefficiently to bone marrow (Eastmond 2000). In addition, benzene has been shown to cause chromosomal aberrations in spleen lymphocytes, bone marrow, and spermatogonial cells in mice (at exposure levels of 36 to 880 mg/kg b.w.) and, in several studies, to increase micronucleus formation in mouse bone marrow and peripheral blood erythrocytes (at 10 to 600 mg/kg b.w.). The comet assay (single-cell gel electrophoresis) has shown DNA lesions in multiple organs in mice and rats (at 2,000 mg/kg b.w.). Ress *et al.* (2002) reported significant increases in micronuclei in mouse bone marrow at all exposure levels of benzene tested (10, 20, and 40 mg/kg b.w.) (see Section 5.3.3 below).

*In vivo* studies in humans occupationally exposed to benzene (at concentrations of 0.1 to 68 $mg/m^3$ in air) have shown chromosomal aberrations in the bone marrow and lymphocytes to be associated with exposure to benzene (ATSDR 1997). The lengths of exposure in these studies ranged from 6 months to greater than 30 years. Chromosome breakage is the most common genetic damage caused by benzene, but chromosomal deletions and alterations also have been noted (Eastmond 2000). One study investigated

specific chromosomal effects, aneusomy of chromosomes 7 and 8 in lymphocytes, in workers exposed to benzene at a median concentration of 31 ppm (99 mg/m$^3$) benzene. The results showed higher incidence of aneusomy on both chromosomes in the exposed workers than in controls (Zhang *et al.* 1999).

### *5.1.3 Summary of benzene genotoxicity*

Numerous studies in animals and humans have shown that benzene is effective in inducing chromosomal aberrations, chromosomal loss, DNA strand breakage, and micronuclei. However, benzene has been shown to be only weakly effective in inducing point mutations. Studies have shown that the genotoxicity of benzene in humans and in human cultured cells is the same as that found in animals and their cultured cells (Whysner 2000). The pattern of benzene genotoxicity is consistent with the patterns for other leukemia-inducing chemicals. Studies currently are under way investigating the mechanism of benzene's genotoxicity, in particular, the role of oxidative stress and non-DNA targets.

## 5.2 Aniline

Genotoxicity data are less extensive for aniline than for benzene. IARC (1987b) reviewed the genotoxicity of aniline but did not present an overall conclusion, because *in vitro* tests gave both positive and negative results, and no *in vivo* test results were available at the time of the review. No more recent reviews of the genotoxicity of aniline are available. More recent individual studies are summarized below.

### *5.2.1* In vitro *assays*

Aniline has not shown genotoxic effects in *S. typhimurium*, either with or without metabolic activation, in *E. coli*, or in *Saccharomyces cerevisiae*. Aniline did not induce DNA damage in primary rat hepatocyte cultures or in Chinese hamster lung fibroblasts. However, aniline induced SCE and chromosomal aberrations in several mammalian cell assays (IARC 1987b). Additional studies have shown results consistent with those reported by IARC: negative results in *S. typhimurium* (at exposure levels of 100 to 6,666 μg/plate) (Haworth *et al.* 1983) and positive results for SCE in rat liver epithelial cells (at concentrations of 100 to 1,000 μM) (Cunningham and Ringrose 1983) and human lymphocytes (at aniline hydrochloride concentrations of 50 to 1,000 μM) (Wilmer *et al.* 1984).

### *5.2.2* In vivo *assays*

*In vivo* assays of aniline have shown mixed results, with positive results for micronucleus formation in mouse bone marrow (only at the highest doses tested, 380 and 1,000 mg/kg b.w.) (George *et al.* 1990, Ashby *et al.* 1991, Westmoreland and Gatehouse 1991), DNA lesions in a number of organs in both rats and mice (at 100 mg/kg b.w.) (Sekihashi *et al.* 2002), and SCE in mice, but negative results for micronucleus formation in mice and DNA lesions in the liver and kidney of rats (IARC 1987b). A study in *Drosophila melanogaster* gave positive results for meiotic nondisjunction (at aniline hydrochloride concentrations of 5% to 15%) and negative results for translocation and sex-linked recessive lethal mutation (at aniline hydrochloride concentrations of 3% to 15%) (Muñoz

and Barnett 1998). Ress *et al.* (2002) reported a weak positive response for micronucleus formation in mice given aniline at doses of 23 and 470 mg/kg b.w. (see Section 5.3.3).

### *5.2.3 Summary of aniline genotoxicity*

Aniline has not been shown to cause point mutations in *in vitro* tests, but it has been shown to cause SCE in *in vitro* tests. Mixed results have been seen for *in vivo* tests, with both positive and negative results for micronucleus formation and DNA lesions in rats and mice.

## 5.3 DAAB

### *5.3.1 Prokaryotic systems*

DAAB (0.1 to 100 µg/plate) was tested in S. *typhimurium* strains TA98, TA100, and TA1537 both with and without rat or hamster liver S9 enzymes for metabolic activation. The results were positive for mutagenicity in strains TA98, TA100, and TA1537 with rat or hamster S9 activation. The results were negative in all strains without metabolic activation and in strain TA1535 both with and without metabolic activation (Zeiger *et al.* 1987). [The fact that DAAB was mutagenic in three of four *S. typhimurium* strains tested, whereas benzene and aniline generally are not mutagenic in these assays (see Sections 5.1 and 5.2, above), indicates that the structure of the parent molecule or free radicals generated in the course of its metabolism may account for additional mechanism(s) of genotoxicity.]

### *5.3.2 Plants*

In a report of the U.S. Environmental Protection Agency Gene-Tox Program, Grant (1982) summarized results for induction of chromosomal aberrations in the common onion (*Allium cepa*) for 148 chemicals. DAAB induced chromosomal aberrations at a concentration of 1,250 ppm.

### *5.3.3 Micronucleus assay*

Ress *et al.* (2002) tested DAAB, benzene, aniline, and a mixture of benzene and aniline for induction of micronuclei in polychromatic erythrocytes (PCEs) in the bone marrow of male B6C3$F_1$ mice (Table 5-1 shows the dose levels and results). The basis for the doses chosen was that DAAB is metabolized essentially into 40% benzene, 47% aniline, and 13% nitrogen. Micronuclei were induced by DAAB, benzene, and the mixture of benzene and aniline. This study provided the first evidence of DAAB-induced chromosomal damage. It is important to note, however, that benzene exposure in humans has been linked to clastogenic events, as indicated by the evidence of increased structural and numerical chromosomal damage in lymphocytes (Zhang *et al.* 1999, Giver *et al.* 2001).

In the comparative study of DAAB, benzene, aniline, and a benzene-aniline mixture (Ress *et al.* 2002), significant increases in the frequency of micronucleated PCEs were reported for all groups of mice exposed to DAAB, benzene, or the mixture; however, DAAB was the strongest inducer of micronuclei at the higher doses. In the mice exposed to aniline, significant increases were noted in the 23-mg/kg (0.25-mmol/kg) and 470-mg/kg (5.05-mmol/kg) dose groups; the authors considered these results to represent a

weak positive response. No differences in micronucleus formation were observed between the mice administered DAAB at 25 mg/kg and those given a 1:1 molar mixure of benzene and aniline equivalent to 25 mg/kg of DAAB. However, in mice given DAAB at 50 or 100 mg/kg, the frequency of micronucleated PCEs was 1.4 to more than 2 times that observed in the groups receiving benzene or the benzene-aniline mixture (Table 5-1). [It is noted that for all exposure groups, the standard error was large relative to the mean.] The authors concluded that DAAB was a potent inducer of micronuclei in mice and that its activity likely was related to that of benzene (a primary metabolite of DAAB).

**Table 5-1. Micronuclei induction in mice (5 per exposure group)**

| Chemical | Dose (mg/kg) | MN-PCE (±SEM) per 1000[a] | % PCE |
|---|---|---|---|
| DAAB | 0 | 0.70 ± 0.25 | 57.0 |
| | 25 | 2.10 ± 0.75* | 61.2 |
| | 50 | 5.00 ± 1.75** | 60.4 |
| | 100 | 9.00 ± 2.45** | 63.4 |
| | | $P < 0.001$[b] | |
| Benzene | 0 | 0.70 ± 0.25 | 57.0 |
| | 10 | 2.40 ± 0.40 | |
| | 20 | 2.50 ± 1.65** | 55.2 |
| | 40 | 6.30 ± 2.10** | 63.9 |
| | | $P < 0.001$[b] | |
| Aniline | 0 | 0.70 ± 0.25 | 57.0 |
| | 12 | 1.20 ± 0.90 | |
| | 23 | 2.60 ± 0.95* | 68.9 |
| | 47 | 1.40 ± 1.05 | 59.7 |
| | 120 | 2.10 ± 0.75 | 64.6 |
| | 470 | 3.30 ± 2.95** | 63.6 |
| | | $P = 0.001$[b] | |
| mixture (Benzene/Aniline) | 0 | 0.70 ± 0.25 | 57.0 |
| | 10/12 | 2.00 ± 1.60 | |
| | 20/23 | 3.00 ± 1.25** | 62.4 |
| | 40/47 | 3.80 ± 1.90** | 65.5 |
| | | $P < 0.001$[b] | |
| Cyclophosphamide (positive control) | 20 | 17.9 ± 2.58 | 57.3 |

Sources: NTP (2002a) and Ress *et al.* (2002).

*$P < 0.005$, **$P \leq 0.001$

[a]Significance of pairwise comparison of treated group to vehicle (corn oil) control. A single control group was used for all test chemicals.

[b]One-tailed Cochran Armitage trend test.

## 5.4 Summary

Few genotoxicity studies of DAAB are available. These studies indicate that DAAB causes gene mutation in *S. typhimurium* with metabolic activation, chromosomal aberrations in the common onion, and micronucleus formation in the bone marrow of mice. Benzene, a major metabolite of DAAB, causes DNA adduct formation in human cells, inhibits RNA synthesis in *in vitro* tests, and induces micronuclei, chromosomal aberrations, and DNA lesions in mice and rats, but generally has not caused point mutations in *in vitro* tests. In addition, studies of workers occupationally exposed to benzene have reported increases in chromosomal aberrations and SCE. Aniline, another major metabolite of DAAB, induces SCE, micronuclei, and DNA lesions in mice and rats, but does not cause point mutations or DNA damage *in vitro*. Recent toxicity studies indicate that DAAB has toxic properties similar to those of its two main metabolites. However, DAAB may be a more potent genotoxicant than either benzene or aniline. DAAB is mutagenic in bacteria and induces micronucleus formation to a greater extent at doses ≥ 50 mg/kg b.w. than do benzene or a mixture of benzene and aniline at molar equivalent doses.

# 6 Other Relevant Data

The NTP sponsored a number of studies to characterize the toxicity and fate of DAAB in laboratory animals (NTP 2002a). As discussed above, DAAB was selected for study based on its potential for worker exposure, its presence as an impurity in foods, drugs, and cosmetics, the fact that it caused gene mutation in *Salmonella typhimurium*, and the lack of adequate toxicological data. It also was selected because its chemical structure suggested that it would be metabolized to benzene and aniline. Benzene is a known human carcinogen (NTP 2002b, IARC 1982a), and aniline was a positive carcinogen in chronic studies with rats (NCI 1978, IARC 1987b, CIIT 1982).

The metabolism and disposition of DAAB were characterized following oral, dermal, or intravenous administration to male and female F344/N rats and male B6C3$F_1$ mice as well as in human liver slices. As a follow-up to observations made in the course of metabolism studies, electron spin resonance studies were conducted to assess the possible formation of the phenyl radical in the course of DAAB metabolism. Toxicity studies were limited to16-day dermal studies using male and female F344/N rats and B6C3$F_1$ mice. Significant findings from each of these studies are described below.

## 6.1 Absorption, distribution, metabolism, and excretion studies

### *6.1.1 Disposition studies*

Studies of the fate of $^{14}$C-labeled DAAB (randomly labeled on the phenyl rings) in rats demonstrated that the radioactivity was readily and nearly completely absorbed from the gastrointestinal tract but was absorbed from skin to a lesser extent (≤ 7%). Application of a much higher dose of dermally applied radiolabeled DAAB did not result in a proportionally higher mass of labeled material absorbed. The absolute amount absorbed was similar for the 2 mg/kg and 20 mg/kg doses. Following absorption via either route, DAAB was rapidly metabolized, and the radioactive metabolites were excreted primarily in urine (NTP 2002a, Mathews and De Costa 1999). Approximately 80% of the radioactivity of an i.v. dose was excreted in urine by male rats within the first 24 hours after administration (Table 6-1). [Comparison of excretion in urine following oral vs. i.v. administration indicates that approximately 90% of the total radioactivity was absorbed from the gastrointestinal tract of rats.] Gastrointestinal absorption was similar in mice and rats, and urine was the primary route of excretion in both species; however, radiolabeled metabolites were excreted less rapidly by mice. Some of the differences in the relative importance of urinary vs. fecal routes of excretion for mice vs. rats also may be attributed to the fact that, for mice held in metabolic chambers, their feces tend to be contaminated with urine. In addition, total recovery of the labeled dose generally was lower in mice. Exhalation as volatile organics and carbon dioxide in the breath of male rats and mice accounted for less than 2% of the dose administered by any route.

**Table 6-1. Excretion of $^{14}C$-labeled DAAB in male rats and male mice**

| Route of administration | Dose | Percent of administered dose (mean ± SD) | | | |
|---|---|---|---|---|---|
| | | Urine | Feces | Breath | Total |
| Male F344/N rats – 24 hours | | | | | |
| Intravenous | 2 mg/kg | 80.1 ± 3.3 | 5.2 ± 0.7 | 0.60 ± 0.08 | 85.9 ± 3.9 |
| Gavage | 20 mg/kg | 75.5 ± 2.0 | 13.7 ± 1.6 | 1.30 ± 0.21 | 90.6 ± 3.0 |
| Dermal | 2 mg/cm$^2$ | 1.44 ± 0.38 | 0.09 ± 0.02 | 0.13 ± 0.03 | 1.66 ± 0.42 |
| Dermal | 20 mg/cm$^2$ | 0.16 ± 0.01 | 0.01 ± 0.00 | 0.05 ± 0.01 | 0.23 ± 0.01 |
| Male F344/N rats – 72 hours (includes cage wash) | | | | | |
| Intravenous | 2 mg/kg | 87.0 ± 1.8 | 7.7 ± 0.4 | 0.67 ± 0.10 | 95.3 ± 1.6 |
| Gavage | 20 mg/kg | 80.3 ± 0.8 | 15.7 ± 0.8 | 1.36 ± 0.22 | 97.5 ± 0.3 |
| Dermal | 2 mg/cm$^2$ | 4.67 ± 1.19 | 0.41 ± 0.08 | 0.36 ± 0.09 | 5.44 ± 1.44 |
| Dermal | 20 mg/cm$^2$ | 0.44 ± 0.03 | 0.05 ± 0.01 | 0.08 ± 0.01 | 0.57 ± 0.02 |
| Male B6C3F$_1$ mice – 24 hours | | | | | |
| Intravenous | 2 mg/kg | 27.2 ± 10.5 | 11.4 ± 3.5 | 0.74 ± 0.31 | 39.7 ± 10.9 |
| Gavage | 20 mg/kg | 44.3 ± 21.8 | 15.2 ± 6.7 | 0.87 ± 0.20 | 60.6 ± 16.9 |
| Dermal | 2 mg/cm$^2$ | 0.59 ± 0.25 | 0.42 ± 0.06 | 0.19 ± 0.14 | 1.20 ± 0.32 |
| Dermal | 20 mg/cm$^2$ | 0.08 ± 0.01 | 0.07 ± 0.06 | 0.04 ± 0.01 | 0.20 ± 0.05 |
| Male B6C3F$_1$ mice – 72 hours (includes cage wash) | | | | | |
| Intravenous | 2 mg/kg | 43.4 ± 6.5 | 22.9 ± 5.6 | 1.02 ± 0.36 | 69.4 ± 5.2 |
| Gavage | 20 mg/kg | 60.9 ± 15.0 | 20.0 ± 10.8 | 1.03 ± 0.27 | 82.2 ± 10.5 |
| Dermal | 2 mg/cm$^2$ | 1.92 ± 1.07 | 2.40 ± 0.84 | 0.48 ± 0.26 | 4.80 ± 0.26 |
| Dermal | 20 mg/cm$^2$ | 0.26 ± 0.01 | 0.36 ± 0.07 | 0.09 ± 0.01 | 0.71 ± 0.08 |

Source: NTP 2002a (Tables A1, A2, A4, A11, A12, and A13).

As evident from the excretion data, DAAB was not retained in tissues. DAAB-derived radioactivity was detected at low levels (< 1% total dose) in the adipose tissue, blood, kidney, liver, muscle, skin, and spleen in male and female rats 24 hours after oral administration of 20 mg/kg b.w. (Table 6-2) (Mathews and De Costa 1999, NTP 2002a). [The highest concentrations of DAAB-derived radioactivity were observed in kidney, but that likely was due to the fact that the primary route of excretion was in urine.]

**Table 6-2. Tissue distribution of radioactivity in F344/N male and female rats**

| Tissue[b] | Male rats[a] | | Female rats[a] | |
|---|---|---|---|---|
| | DAAB equivalents in tissue (ng/g) | Dose in total tissue (%) | DAAB equivalents in tissue (ng/g) | Dose in total tissue (%) |
| Adipose | 438 ± 113[c] | 0.155 ± 0.041 | 803 ± 443 | 0.273 ± 0.150 |
| Blood | 1,380 ± 80 | 0.361 ± 0.016 | 1,370 ± 300 | 0.346 ± 0.075 |
| Kidney | 1,910 ± 150 | 0.067 ± 0.006 | 2,430 ± 440 | 0.084 ± 0.016 |
| Liver | 912 ± 30 | 0.184 ± 0.002 | 1,090 ± 140 | 0.182 ± 0.025 |
| Muscle | 123 ± 11 | 0.297 ± 0.030 | 137 ± 17 | 0.319 ± 0.039 |
| Skin | 229 ± 11 | 0.196 ± 0.007 | 253 ± 43 | 0.210 ± 0.034 |
| Spleen | 671 ± 25 | 0.007 ± 0.001 | 976 ± 184 | 0.012 ± 0.002 |

Source: NTP 2002a (Table A9).

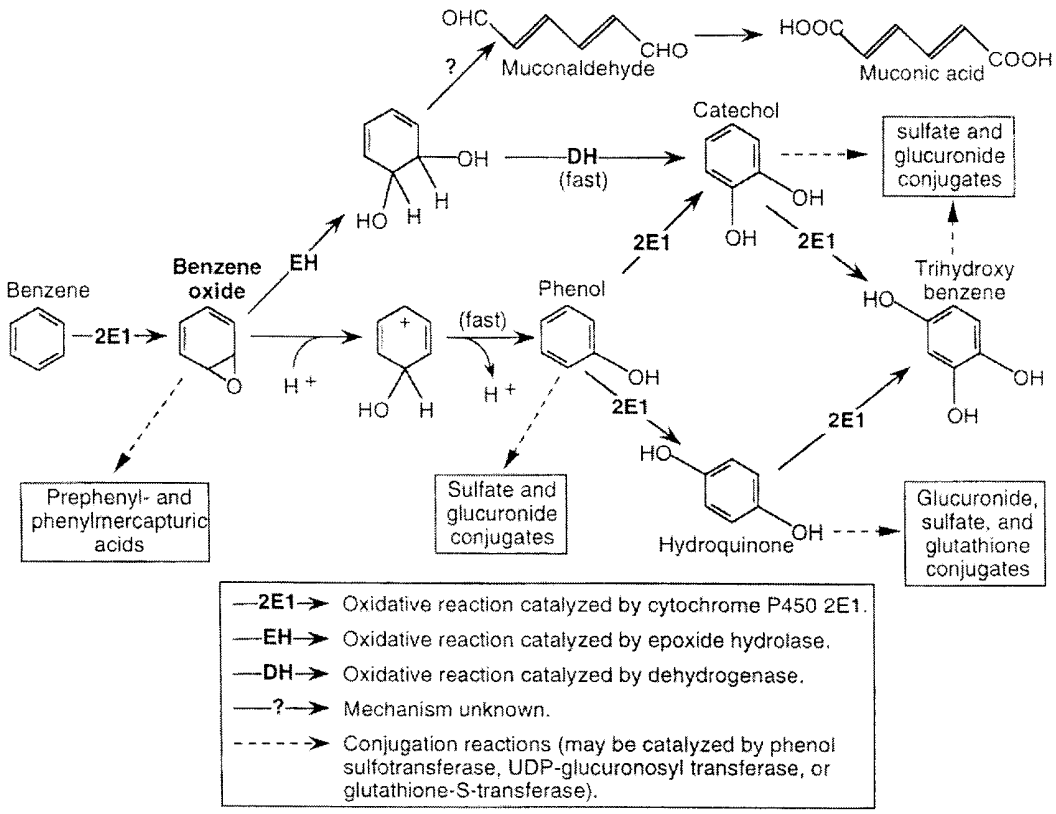
[a]Results obtained from three male and four female rats.

[b]Tissues were collected 24 hours after a single gavage of 20 mg/kg [$^{14}$C]DAAB.

[c]Mean ± SD.

### *6.1.2 Metabolism of benzene*

Many of the urinary metabolites detected after administration of DAAB to rats and mice were known metabolites of benzene. The metabolism of benzene, reviewed recently by Lovern *et al.* (2001), is illustrated in Figure 6-1. Benzene initially is oxidized to benzene oxide, which may rearrange spontaneously to form phenol or may be converted enzymatically to a 1,2-dihydrodiol intermediate by epoxide hydrolase. Phenol may be oxidized by cytochrome P-450 2E1 to hydroquinone or catechol. Catechol also may be formed by dehydrogenation of the 1,2-dihydrodiol intermediate. The 1,2-dihydrodiol intermediate may give rise to muconic acid via the postulated intermediate muconaldehyde.




Source: Lovern *et al.* 2001.

**Figure 6-1. Metabolism of benzene**

The metabolism of benzene has been shown to be dose-dependent (Mathews *et al.* 1998). When benzene was administered at 100 mg/kg b.w., approximately 50% of the dose was exhaled unchanged, whereas at doses of 0.5 mg/kg or less, only about 2% was exhaled unchanged. In each case the remainder of the dose was excreted primarily in urine in the form of a number of metabolites. The major metabolites formed varied among rats, mice, and hamsters. Phenyl sulfate accounted for 64% to 73% of the dose excreted by rats. Phenyl sulfate (24% to 32%) and hydroquinone glucuronide (27% to 32%) were the major metabolites excreted by mice. Of the minor metabolites observed, mice formed more muconic acid than did rats (15% vs. 7%), and hamsters formed still more muconic acid (19% to 31%). The other major metabolite excreted by hamsters was hydroquinone glucuronide (24% to 29%). Minor metabolites observed in one or more species were prephenylmercapturic acid [the nonaromatic product of the oxirane ring opening of benzene oxide with thiol], phenylmercapturic acid, hydroquinone, phenol glucuronide, hydroquinone sulfate, and phenol. Trihydrobenzene and catechol sulfate were detected in hamsters only (Mathews *et al.* 1998).

### 6.1.3 Metabolism of aniline

When rats and mice were administered DAAB orally, the urine contained several metabolites also observed as metabolites of aniline. The urinary metabolites of aniline in F344 rats (sex not specified) and $B6C3F_1$ mice (sex not specified) were identified by HPLC 24 hours after intraperitoneal (i.p.) injection of $^{14}C$-labeled aniline (McCarthy *et al.* 1985). The percentages of urinary metabolites for the lower dose administered to rats (50 mg/kg b.w.; 0.54 mmol/kg) are reported in Table 6-4, and those for the lower dose administered to mice (100 mg/kg b.w.; 1.07 mmol/kg) are reported in Table 6-5. Rats and mice also received doses of 250 mg/kg (2.68 mmol/kg) and 500 mg/kg (5.37 mmol/kg), respectively. A total of 8 metabolites were identified: aniline, *p*(4)-aminophenyl glucuronide, *p*(4)-aminophenyl sulfate, *o*(2)-aminophenyl sulfate, *p*(4)-acetamidophenol, *p*(4)-acetamidophenyl glucuronide, *p*(4)-acetamidophenyl sulfate, and *o*(2)-acetamidophenyl sulfate. HPLC chromatograms of urine from mice showed three unidentified peaks, representing 23.6% of the low dose and 25.7% of the high dose of radiolabeled aniline. Rat urine contained only one of the unknown metabolites, representing 0.5% of the total radioactivity.

Following oral administration of aniline to rats, sulfation was the major metabolic pathway for elimination, but the degree of sulfation varied with dose. *p*(4)-Acetamidophenyl sulfate accounted for > 70% of the urinary metabolites at a dose of 50 mg/kg, but only 30% at 250 mg/kg. Rats receiving the high dose excreted more *p*(4)-acetamidophenyl glucuronide and unconjugated metabolites (McCarthy *et al.* 1985).

### 6.1.4 Metabolism of DAAB

Benzene, aniline, and metabolites of benzene and aniline were detected in the blood of rats orally dosed with DAAB, and these metabolites were excreted in urine of dosed rats and mice (Mathews and De Costa 1999). The profile of DAAB metabolites observed in rats and mice was similar following dermal, oral, or intravenous administration. The metabolic conversion of DAAB to metabolites of benzene is consistent with the pathway illustrated in Figure 6-1.

#### 6.1.4.1 Metabolites of DAAB in exhaled breath

Following oral administration of $^{14}C$-labeled DAAB to rats and mice, benzene was the only radiolabeled product detected in exhaled breath, with 1.30% and 0.87% recovered 24 hours after gavage exposure in rats and mice, respectively (Mathews and De Costa 1999).

#### 6.1.4.2 Common metabolites of DAAB, benzene, and aniline in blood

Within 15 minutes following oral administration, the predominant DAAB equivalents in blood of rats were known metabolites of benzene (i.e., phenol, hydroquinone glucuronide, muconic acid, prephenylmercapturic acid, phenol glucuronide, and phenyl sulfate) and aniline (i.e., 4-acetamidophenyl sulfate). Both benzene and aniline were detected at all time points, with peak concentrations at 1.0 and 0.5 hour, respectively. Of the benzene metabolites present, phenyl sulfate occurred at the highest concentration in both males and females. Muconic acid also was a major metabolite in both males and females, although in males, hydroquinone glucuronide was present at a higher

concentration than muconic acid. In both sexes, benzene was one of the four metabolites with the highest concentrations. Table 6-3 shows the metabolites observed at 6 hours after administration. No analysis was reported for metabolites in blood of mice.

**Table 6-3. Metabolites of DAAB observed in blood of male and female rats following oral dosing**

| | Male rats[a,b] | Female rats[a,b] |
|---|---|---|
| **Metabolites of benzene** | | |
| Benzene | 51 ± 13 | 53 ± 13 |
| Phenol | 15 ± 15 | 2 ± 2 |
| Hydroquinone glucuronide | 89 ± 38 | 73 ± 15 |
| Muconic acid | 32 ± 12 | 102 ± 21 |
| Prephenylmercapturic acid | 22 ± 12 | 19 ± 3 |
| Phenol glucuronide | 14 ± 6 | 8 ± 4 |
| Phenyl sulfate | 210 ± 36 | 162 ± 40 |
| **Metabolites of aniline** | | |
| Aniline | 27 ± 12 | 10 ± 7 |
| 4-Acetamidophenyl sulfate | 366 ± 82 | 305 ± 13 |

Source: NTP 2002a (Table A10).

[a]Mean (± SD) blood concentration for rats in ng-Equivalents per g of blood 6 h after oral dosing.

[b]Metabolites of DAAB were identified in the blood of F344/N male and female rats after gavage administration of 20 mg/kg [$^{14}$C]DAAB.

Sabourin *et al.* (1989) profiled the metabolites of benzene in the blood of male F344 rats following oral exposure at doses of 1, 10, and 200 mg/kg b.w. The authors reported blood concentrations of two water-soluble benzene metabolites, which they described as representing a putative detoxification pathway (phenyl sulfate) and a putative toxification pathway (muconic acid). The highest concentration of each metabolite was present in blood collected at 0.5 hour after administration (the first time point), and both phenyl sulfate and muconic acid concentrations had decreased to baseline levels by 8 to 12 hours after administration. Area-under-the-curve analyses indicated that phenyl sulfate was the predominant metabolite, with smaller amounts of prephenylmercapturic acid and muconic acid.

The metabolites detected in the blood of rats exposed to 20 mg/kg of DAAB were qualitatively similar to those that resulted from oral exposure to benzene, with phenyl sulfate being the benzene-derived metabolite present in the highest concentration in blood of both male and female rats (Mathews and De Costa 1999). Muconic acid also was a major metabolite in both male and female rats exposed to DAAB, although in the blood of male rats, hydroquinone glucuronide was present at a higher concentration than was muconic acid. The blood of both male and female rats exposed to 20 mg/kg DAAB also contained benzene as one of the four metabolites with the highest concentration.

*6.1.4.3 Common metabolites of DAAB, benzene, and aniline in urine*

Urinary metabolites identified and quantified in the urine of rats and mice administered [$^{14}$C]benzene, [$^{14}$C]aniline, or [$^{14}$C]DAAB are listed in Tables 6-4 (rats) and 6-5 (mice). The pattern of metabolites excreted in urine indicated some evidence of species-specific differences in metabolism; however, DAAB metabolites common to both benzene and aniline were observed in the urine of both rats and mice. A quantitative comparison of these metabolites is difficult since recovery data were calculated in different ways, e.g., for rats Mathews *et al.* (1998) (benzene) and McCarthy *et al.* (1985) (aniline) reported results for metabolites as percentage of total urinary radioactivity recovered while Mathews and De Costa (1999) (DAAB) expressed results in terms of the percentage of the administered dose. The percentages for mice were based on total urinary radioactivity recovered for all three chemicals. The doses of chemicals also differed between mice and rats. In addition, the assay methods varied among the three studies; although HPLC was used in all three studies, the type of column and eluting reagents differed. It is not clear whether the differences in methodology contributed to differences in metabolite profiles among the three studies. [For these reasons, the data are best suited to qualitative comparisons.]

Mathews *et al.* (1998) administered benzene to rats at 0.02, 0.1, 0.5, 10 (results shown in Table 6-4), and 100 mg/kg b.w. and to mice at 0.1 (results shown in Table 6-5) and 100 mg/kg b.w. The profile of metabolites (rank order by amount recovered) was qualitatively similar across doses for rats; phenyl sulfate always represented the largest percentage of administered radioactivity, with prephenylmercapturic acid second or third in abundance. The results for mice varied more with the dose. At 0.1 mg/kg (see Table 6-5), hydroquinone glucuronide accounted for more radioactivity than did phenyl sulfate; however, at 100 mg/kg, the order was reversed, with 31.68% ± 0.46% phenyl sulfate and 26.49% ± 1.66% hydroquinone glucuronide.

McCarthy *et al.* (1985) administered aniline at doses of 50 mg/kg (shown in Table 6-4) and 250 mg/kg to rats and 100 mg/kg (shown in Table 6-5) and 500 mg/kg to mice. Percentages of urinary metabolites generally were similar for mice at both doses. The results for rats varied more with the dose; for example, comparing the results at the low and high doses, 4-acetamidophenyl sulfate decreased from 71.8% to 30.3%, and 4-acetamidophenyl glucuronide increased from 0.0% to 11.6%.

The results reported for DAAB by Mathews and De Costa (1999) were for a single dose level of 20 mg/kg in both rats and mice. Among the metabolites of DAAB, prephenylmercapturic acid, phenol glucuronide, and phenyl sulfate were observed only in the rat, and phenol was detected only in mouse urine. Rats excreted only a single metabolite of aniline, 4-acetamidophenyl sulfate, in urine, whereas mice excreted five aniline metabolites in urine (Mathews and De Costa 1999).

The metabolism studies showed that rats and mice metabolize DAAB almost exclusively to benzene, aniline, and their known metabolites. Based on the proposed mechanism of decomposition, DAAB is expected to yield approximately 40% benzene, 47% aniline, and 13% nitrogen. Within 24 hours after administration, the percentages of the oral dose of radiolabeled DAAB excreted in the urine as benzene metabolites were 30% in rats and

22% in mice; the percentages eliminated as aniline metabolites were 32% in rats and 35% in mice. In rats, the most prevalent urinary metabolite of both benzene and DAAB was phenyl sulfate, whereas in mice, it was hydroquinone glucuronide. Two metabolites of benzene, hydroquinone glucuronide and muconic acid, were common to both species. 4-Acetamidophenyl conjugates were the most common aniline-derived metabolites of both aniline and DAAB. In rats, the sulfate conjugate was the most common, whereas in mice, the glucuronide conjugate predominated.

**Table 6-4. Urinary metabolites (0 to 24 hours) as percent of administered dose or of total urinary radioactivity in F344 rats administered benzene, aniline, or DAAB**

| Urinary metabolite | Metabolites of benzene[a] | Metabolites of aniline[b] | Metabolites of DAAB[c] |
|---|---|---|---|
| Phenyl sulfate | 70.32 ± 0.49 | | 15.0 ± 0.6 |
| Prephenylmercapturic acid | 11.24 ± 1.66 | | 2.9 ± 0.4 |
| Phenol | 4.20 ± 0.16 | | |
| Hydroquinone glucuronide | 3.77 ± 0.52 | | 6.7 ± 0.3 |
| Muconic acid | 3.62 ± 1.28 | | 2.4 ± 0.3 |
| Phenol glucuronide | 1.67 ± 0.20 | | 2.3 ± 0.9 |
| Phenylmercapturic acid | 1.52 ± 0.23 | | |
| Hydroquinone | 0.54 ± 0.07 | | |
| Hydroquinone sulfate | < 0.05 | | |
| 4-Acetamidophenyl sulfate | | 71.8 | 32.3 ± 1.5 |
| 4-Aminophenyl sulfate | | 10.0 | |
| 2-Aminophenyl sulfate | | 9.4 | |
| 2-Acetamidophenyl sulfate | | 1.2 | |
| 4-Acetamidophenol | | 1.0 | |

[a]Mathews *et al.* (1998); 10.0 mg benzene/kg b.w. (0.13 mmol/kg) administered by gavage; mean ± SD (n = 4); percentage of urinary radioactivity appearing as each metabolite in the 0 to 24 hour pooled urine collection.

[b]McCarthy *et al.* (1985); 50 mg aniline/kg b.w. (0.54 mmol/kg) administered by i.p. injections; mean value (SD, and number of rats not reported); percentage of total radioactivity recovered in the 0 to 24 hour collection for each metabolite.

[c]Mathews and De Costa (1999); 20 mg DAAB/kg b.w. (0.10 mmol/kg) administered by gavage; mean ± SD (n = 4); percentage of the dose recovered in 0 to 24 hour urine collection for each metabolite.

**Table 6-5. Urinary metabolites (0 to 24 hours) as percent of total urinary radioactivity in B6C3F$_1$ mice administered benzene, aniline, or DAAB**

| Urinary metabolite | Metabolites of benzene[a] | Metabolites of aniline[b] | Metabolites of DAAB[c] |
|---|---|---|---|
| Hydroquinone glucuronide | 31.97 ± 1.31 | | 13.0 ± 3.1 |
| Phenyl sulfate | 23.69 ± 0.61 | | |
| Muconic acid | 15.32 ± 1.93 | | 3.9 ± 0.9 |
| Phenylmercapturic acid | 3.71 ± 1.31 | | |
| Phenol glucuronide | 3.08 ± 0.09 | | |
| Phenol | 2.95 ± 0.29 | | 4.7 ± 0.5 |
| Hydroquinone sulfate | 2.84 ± 0.34 | | |
| Prephenylmercapturic acid | 2.03 ± 0.28 | | |
| Hydroquinone | 1.40 ± 0.17 | | |
| 4-Acetamidophenyl glucuronide | | 31.2 | 23.6 ± 2.3 |
| 2-Aminophenyl sulfate | | 16.5 | 1.5 ± 2.8 |
| 4-Acetamidophenol | | 9.0 | 0.8 ± 1.0 |
| 2-Acetamidophenyl sulfate | | 5.8 | ND |
| 4-Aminophenyl glucuronide | | 1.6 | |
| 4-Acetamidophenyl sulfate | | | 4.5 ± 0.8 |
| 2-Acetamidophenol | | | 5.0 ± 1.1 |

[a]Mathews *et al.* 1998; 0.1 mg benzene/kg b.w. (0.0013 mmol/kg) administered by gavage; mean ± SD (n = 4); percentage of urinary radioactivity appearing as each metabolite in the 0 to 24 hour pooled urine collection.

[b]McCarthy *et al.* 1985; 100 mg aniline/kg b.w. (1.07 mmol/kg) administered by i.p. injection; mean value (SD and number of mice not reported); percentage of total radioactivity recovered in the 0 to 24 hour collection for each metabolite.

[c]Mathews and De Costa 1999; 20 mg DAAB/kg b.w. (0.10 mmol/kg) administered by gavage; mean ± SD (n = 4); percentage of urinary radioactivity appearing as each metabolite in the 0 to 24 hour pooled urine collection

ND = not detected.

Sabourin *et al.* (1989) also profiled the metabolites of benzene in the urine of F344 rats following oral administration at 1, 10, and 200 mg/kg b.w. The profile of water-soluble benzene metabolites in 24-hour urine samples was similar to that found in blood. Phenyl sulfate constituted approximately 70% of the total metabolites, followed by either prephenylmercapturic acid or muconic acid, depending on the dose level.

#### *6.1.4.4 Studies with human liver slices*

A study of the metabolism of $^{14}$C-labeled DAAB by human liver slices demonstrated that DAAB could be cleaved to yield metabolites of benzene and aniline (Mathews and De Costa 1999). After 5 hours of incubation, the medium still contained about 90% of the

radioactivity, but only 1% to 2% of that radioactivity represented metabolites of DAAB. Metabolites previously characterized in urine (4-acetamidophenyl sulfate, phenyl sulfate, aniline, and hydroquinone glucuronide) were confirmed in the media samples. In a similar study of benzene using liver slices and liver microsomal fractions from humans, rats, and mice, a similar spectrum of benzene metabolites was detected (Brodfuehrer *et al.* 1990).

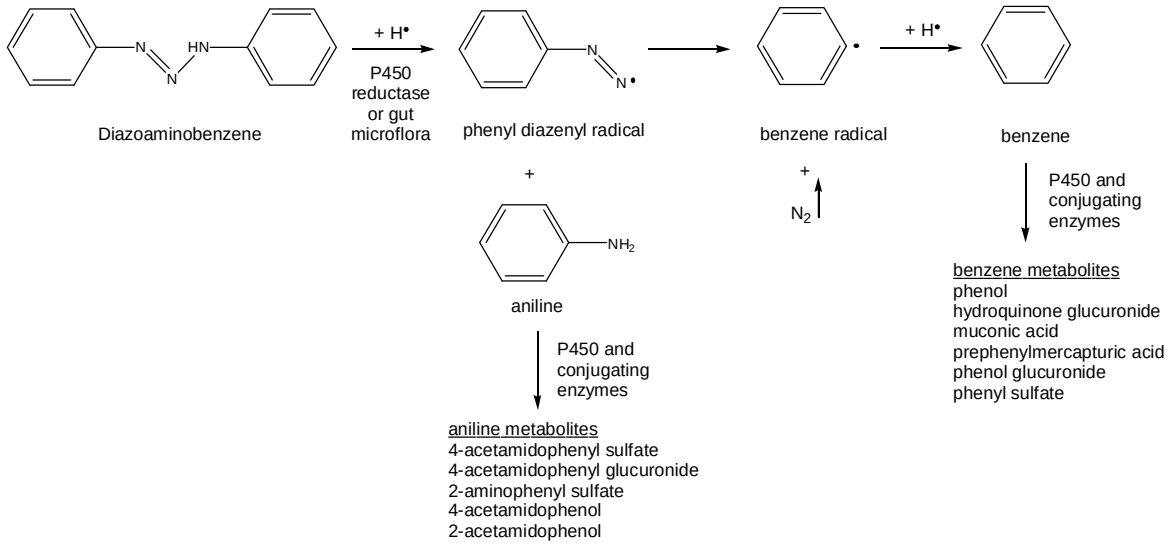
#### *6.1.4.5 Proposed metabolic pathway for DAAB*

As described above, the profile of DAAB metabolites observed in rats and mice was similar following dermal, oral, or intravenous administration. Metabolites detected in the blood and urine of rats or mice exposed to DAAB included those previously characterized for benzene (i.e., muconic acid, prephenylmercapturic acid, hydroquinone glucuronide, phenol glucuronide, phenyl sulfate, and phenol) and aniline (i.e., 2-aminophenyl sulfate, 4-acetamidophenyl glucuronide, 4-acetamidophenyl sulfate, 2-acetamidophenyl sulfate, 4-acetamidophenol, and 2-acetamidophenol).

Matthews and De Costa (1999) proposed that DAAB is cleaved reductively to form aniline and phenyl diazenyl radicals. The diazenyl radical fragments on further reaction ultimately yield nitrogen and benzene. The benzene and aniline are subsequently metabolized to form the metabolites detected in blood and urine of exposed animals (Figure 6-2). The metabolic conversion of DAAB to metabolites of benzene with benzene oxide as the initial metabolic step is consistent with the pathway illustrated in Figure 6-1. Metabolites of DAAB in blood of rats (Table 6-3), urine of rats (Table 6-4), and urine of mice (Table 6-5) included hydroquinone, muconic acid, and phenylmercapturic acid, which all share benzene oxide as a common intermediate in the pathway proposed by Lovern *et al.* (2001).

The involvement of cytochrome P-450 in the metabolism of DAAB or the further metabolism of benzene and aniline derived from DAAB was supported by data obtained from rats pretreated with 1-aminobenzotriazole (ABT) prior to oral administration of DAAB (Mathews and De Costa 1999). Urinary excretion of DAAB-derived radiolabel during the first 8 hours decreased from about 50% for non-ABT-treated rats to about 12% in ABT-treated rats. This change in metabolism was accompanied by an increase in the amount of unchanged benzene exhaled in the breath. Urinary excretion of the aniline metabolite 4-acetamidophenyl sulfate was delayed in rats pretreated with ABT; the majority of the metabolite was excreted in the 8-to-24-hour collection rather than in the first 8 hours following administration. The existence of transient free-radical metabolites of DAAB was confirmed in both *in vitro* and *in vivo* experiments using electron spin resonance (ESR) (see Section 6.1.4.6).

Because the micronucleus assays described in Section 5 were conducted with mouse bone marrow cells, metabolism of DAAB within the bone marrow could be a factor in the local effects of DAAB metabolites, including the phenyl radical. Although no studies on metabolism of DAAB by bone marrow were located, bone marrow cells do produce cytochrome P-450 (Heidel *et al.* 1998, Bernauer *et al.* 1999).




Source: Mathews and DeCosta 1999.

**Figure 6-2. Proposed pathway for the metabolism of DAAB**

*6.1.4.6 Electron spin resonance studies*

Because the metabolic pathway proposed for DAAB was thought to generate free radicals, a series of ESR studies were designed and conducted using the free radical trapping agent 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) (Kadiiska *et al.* 2000). *In vitro* studies using rat hepatic microsomes incubated with DAAB indicated that NADPH was required for the formation of the DMPO-phenyl adduct, indicative of free-radical formation. The phenyl radical also was formed in microsomes incubated with the mechanism-based P-450 inhibitor ABT and in the presence of carbon monoxide, indicating that interaction with the heme prosthetic group of P-450 is not required for initial cleavage of DAAB, although further metabolism of benzene and aniline do require P-450. Formation of the DMPO-phenyl adduct also was catalyzed by recombinant human NADPH P-450 reductase.

The possibility that metabolism of DAAB might be catalyzed by microbes in the gastrointestinal tract was investigated in an experiment that used anaerobic incubations of cecal contents isolated from rats (Kadiiska *et al.* 2000). Low levels of the DMPO-phenyl adduct were detected, suggesting that intestinal microbes could play a role in the metabolism of any DAAB that was not absorbed from the gastrointestinal tract before reaching the cecum.

Free-radical formation was demonstrated *in vivo* in studies in which intact bile duct-cannulated rats were administered DAAB via intragastric intubation and DMPO by i.p. injection. DMPO reacted with the phenyl radical, creating a more stable product that was collected in bile and characterized by ESR spectroscopy. The ESR spectrum was consistent with the formation of a DMPO-phenyl adduct, thus indicating that DAAB was absorbed into the systemic circulation and that the free radical was formed internally (Kadiiska *et al.* 2000).

ESR studies provide evidence that DAAB is reductively cleaved by hepatic enzymes to form phenyl radicals (Figure 6-2). *In vitro* detection of the phenyl radical required the full complement of microsomes or P-450 reductase, NADPH, DAAB, and DMPO (which was used to "trap" the phenyl radical). Addition of P-450 inhibitors, either carbon monoxide or ABT, did not alter the formation of the DMPO-phenyl adduct, indicating that the heme moiety of P-450 is not required for biotransformation. These results suggest that P-450 reductase, rather than the hemoprotein of P-450, catalyzes cleavage of the triazeno linkage, resulting in the formation of aniline and a phenyl diazenyl radical. The phenyl diazenyl radical fragments to form nitrogen and a phenyl radical, which in turn is reduced to benzene.

## 6.2 Short-term toxicity studies in rats and mice

The dermal route was selected for 16-day toxicity studies because it is the major route of exposure in occupational and nonoccupational circumstances (NTP 2002a). However, exposure to DAAB also can occur orally through ingestion of food and drugs. In the disposition study described above (NTP 2002a), the application site was protected from grooming, and dermal absorption accounted for a maximum of 7% of the dose when DAAB was applied at 2 mg/cm$^2$. In contrast, the application site in the 16-day toxicity studies was not protected, allowing for exposure to DAAB both dermally and orally through grooming by treated animals.

In these studies, groups of 5 male and 5 female F344/N rats and B6C3F$_1$ mice received dermal applications of 0, 12.5, 25, 50, 100, or 200 mg DAAB/kg b.w. in acetone, five days per week for 16 days. DAAB was not lethal to rats at any of the concentrations tested. In contrast, most male mice administered 50 mg/kg or more and 3 female mice administered 200 mg/kg died in the second week of the study. A decline in body weight gain was apparent in male and female rats and mice. Statistically significant dose-dependent decreases in absolute and relative thymus weights were observed in male and female rats and mice. Statistically significant increases in relative heart weights in male and female rats and mice also may have been DAAB-related. Relative spleen and liver weights were significantly increased in male and female rats, but not in mice, and relative kidney weights were significantly increased in male rats and male and female mice.

### *6.2.1 Hematologic effects of DAAB*

Hematology data for male and female rats and mice administered DAAB in the NTP (2002a) study are summarized in Tables 6-6a,b (rats) and 6-7a,b (mice). In both sexes of both species, clinical pathology data indicated a DAAB-related methemoglobinemia. Heinz-body formation also was increased and was considered to be related to DAAB exposure. Further, there was a treatment-related decrease in erythroid mass, evidenced by a decrease in hematocrit, hemoglobin, and erythrocyte counts, suggesting a developing anemia. The erythron decrease was accompanied by an increased bone marrow response, as indicated by increased reticulocytes in male and female rats that received DAAB at doses of 50 mg/kg and higher and in all DAAB-exposed female mice. The frequency of nucleated erythrocytes was not increased in male mice, possibly because of poor survival at the three higher doses. In mice only, hemoglobin concentrations were increased in the higher-dose females. This latter response appears inappropriate, compared with other

estimates of red-cell mass, and may have been a spurious result related to the increased number of Heinz bodies. Associated with the anemia in mice was an increase in mean cell hemoglobin concentrations in both sexes, which would be consistent with intravascular hemolyses.

### *6.2.2 Dermal effects*

Gross observations at necropsy revealed significant thickening of the skin at the site of application in all animals dermally exposed to DAAB. Microscopically, this corresponded to hyperplasia of the epidermis and hair follicles, which was evident in all dosed groups (Tables 6-8 to 6-11). Proliferation of hair follicles at the application site was a particularly prominent change of marked severity in the higher-dose groups. In some cases, this lesion was characterized by an extensive area of proliferation containing an increased density of hair follicles. This sometimes formed a raised, plaque-like lesion with a scalloped surface due to coalescence of dilated follicles containing multiple hair shafts. In other areas the interfollicular epidermis was thickened with variable cystic or hyaline-type degeneration in the stratum corneum. A slight infiltration of mixed inflammatory cells accompanied the hyperplastic change. Focal epidermal ulceration at the application site was present in some female mice in the higher-dose groups.

### *6.2.3 Nonneoplastic lesions*

A number of internal nonneoplastic lesions were observed and considered to be related to chemical treatment (Tables 6-8 to 6-11). Lymphoid atrophy of the thymus (a depletion of cortical lymphocytes) was a common lesion; it increased from mild to moderate or marked severity with increasing dose in rats and mice of both sexes and was correlated with reduced thymus weight. A similar adverse effect of treatment on lymphoid tissue was indicated by a loss of mesenteric lymph tissue in rats and mice and mandibular lymph tissue in mice at the highest dose in both sexes. Presumably as a response to anemia, increased incidences of hematopoietic cell proliferation of generally mild severity occurred in the splenic red pulp of DAAB-exposed rats and mice and were correlated with increased spleen weight.

**Table 6-6a. Hematology data for male rats dermally exposed to DAAB for 16 days (mean ± SE)**

| Parameter | Vehicle control | Dose (mg/kg b.w.) | | | | |
|---|---|---|---|---|---|---|
| | | 12.5 | 25 | 50 | 100 | 200 |
| n[a] | 3 | 1[c] | 1[c] | 4 | 2 | 0 |
| Hematocrit (%) | 44.6 ± 1.4 | 42.3 | 43.6 | 41.3 ± 0.7* | 41.5 ± 0.6 | nd |
| Hemoglobin (g/dL) | 15.4 ± 0.5 | 14.5 | 14.6 | 14.0 ± 0.2* | 14.1 ± 0.1 | nd |
| Erythrocytes ($10^6$/μL) | 7.73 ± 0.23 | 7.51 | 7.77 | 7.26 ± 0.11 | 7.18 ± 0.11 | nd |
| Reticulocytes ($10^6$/μL) | 0.29 ± 0.04 | 0.35 | 0.40 | 0.42 ± 0.02* | 0.55 ± 0.03** | nd |
| Nucleated erythrocytes/ 100 leukocytes | 1.67 ± 0.67 | 1.00 | 1.00 | 3.75 ± 1.38 | 1.00 ± 0.00 | nd |
| Mean cell volume (fL) | 58.0 ± 0.0 | 56.0 | 56.0 | 57.3 ± 0.3 | 58.0 ± 0.0 | nd |
| Mean cell hemoglobin (pg) | 20.0 ± 0.0 | 19.3 | 18.8 | 19.4 ± 0.2* | 19.7 ± 0.2 | nd |
| Mean cell hemoglobin concentration (g/dL) | 34.6 ± 0.1 | 34.3 | 33.5 | 34.0 ± 0.3 | 34.0 ± 0.3 | nd |
| Methemoglobin (% hemoglobin) | 0.85 ± 0.05[b] | 1.40 | 2.10 | 3.28 ± 0.09 | 4.00 ± 0.90 | nd |
| Heinz bodies ($10^3$/μL) | 0 | 0 | 0 | 2 ± 2 | 8 ± 8 | nd |

Source: NTP 2002a (Table B7).

*$P \leq 0.05$, **$P \leq 0.01$, compared with the vehicle control group by Dunn's or Shirley's test, using unrounded data.

[a]Five blood samples were collected for analysis, but some were lost because of specimen clotting.

[b]n = 2.

[c]No standard error calculated.

nd = No data due to 100% mortality.

**Table 6-6b. Hematology data for female rats dermally exposed to DAAB for 16 days (mean ± SE)**

| Parameter | Vehicle control | Dose (mg/kg b.w.) | | | | |
|---|---|---|---|---|---|---|
| | | 12.5 | 25 | 50 | 100 | 200 |
| n[a] | 4 | 5 | 5 | 4 | 5 | 5 |
| Hematocrit (%) | 45.3 ± 1.7 | 44.4 ± 1.7 | 42.8 ± 1.1 | 40.7 ± 1.2* | 39.7 ± 1.1** | 39.2 ± 0.6** |
| Hemoglobin (g/dL) | 15.6 ± 0.6 | 15.2 ± 0.5 | 14.5 ± 0.4 | 13.8 ± 0.3** | 13.5 ± 0.3** | 13.5 ± 0.2** |
| Erythrocytes ($10^6$/μL) | 7.58 ± 0.32 | 7.69 ± 0.32 | 7.51 ± 0.20 | 7.14 ± 0.22 | 6.74 ± 0.14* | 6.63 ± 0.11* |
| Reticulocytes ($10^6$/μL) | 0.15 ± 0.02 | 0.27 ± 0.06 | 0.21 ± 0.02 | 0.27 ± 0.03* | 0.33 ± 0.03** | 0.32 ± 0.06** |
| Nucleated erythrocytes/100 leukocytes | 0.25 ± 0.25 | 0.80 ± 0.37 | 0.80 ± 0.20 | 1.25 ± 0.48 | 3.00 ± 1.41* | 6.40 ± 2.54* |
| Mean cell volume (fL) | 60.0 ± 0.4 | 57.8 ± 0.4 | 56.8 ± 0.2** | 57.3 ± 0.5* | 58.8 ± 0.6 | 59.2 ± 0.7 |
| Mean cell hemoglobin (pg) | 20.6 ± 0.3 | 19.8 ± 0.2 | 19.4 ± 0.1* | 19.4 ± 0.3* | 20.0 ± 0.2 | 20.3 ± 0.2 |
| Mean cell hemoglobin concentration (g/dL) | 34.4 ± 0.3 | 34.4 ± 0.3 | 34.0 ± 0.2 | 34.0 ± 0.5 | 34.0 ± 0.2 | 34.4 ± 0.3 |
| Methemoglobin (% hemoglobin) | 0.65 ± 0.13 | 1.44 ± 0.11* | 2.26 ± 0.16** | 3.55 ± 0.36* | 4.56 ± 0.28** | 5.08 ± 0.69** |
| Heinz bodies ($10^3$/μL) | 0 | 0 | 0 | 2 ± 2 | 3 ± 2 | 12 ± 4** |

Source: NTP 2002a (Table B7).

*$P \leq 0.05$, **$P \leq 0.01$, compared with the vehicle control group by Dunn's or Shirley's test, using unrounded data.

[a]Five blood samples were collected for analysis, but some were lost because of specimen clotting.

**Table 6-7a. Hematology data for male mice dermally exposed to DAAB for 16 days (mean ± SE)**

| Parameter | Vehicle control | Dose (mg/kg b.w.) | | | | |
|---|---|---|---|---|---|---|
| | | 12.5 | 25 | 50 | 100 | 200 |
| n[a] | 5 | 4 | 5 | 1[b] | 0 | 0 |
| Hematocrit (%) | 50.0 ± 1.6 | 47.0 ± 1.3 | 46.2 ± 0.6* | 41.8 | nd | nd |
| Hemoglobin (g/dL) | 17.0 ± 0.6 | 16.3 ± 0.3 | 16.5 ± 0.3 | 16.7 | nd | nd |
| Erythrocytes ($10^6$/μL) | 10.58 ± 0.35 | 9.97 ± 0.26 | 9.80 ± 0.13 | 9.08 | nd | nd |
| Reticulocytes ($10^6$/μL) | 0.16 ± 0.03 | 0.13 ± 0.02 | 0.25 ± 0.03 | 0.22 | nd | nd |
| Nucleated erythrocytes/100 leukocytes | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 | nd | nd |
| Mean cell volume (fL) | 47.2 ± 0.2 | 47.3 ± 0.3 | 47.0 ± 0.0 | 46.0 | nd | nd |
| Mean cell hemoglobin (pg) | 16.1 ± 0.1 | 16.4 ± 0.3 | 16.8 ± 0.2* | 18.4 | nd | nd |
| Mean cell hemoglobin concentration (g/dL) | 34.0 ± 0.2 | 34.7 ± 0.7 | 35.7 ± 0.4* | 40.0 | nd | nd |
| Methemoglobin (% hemoglobin) | 0.66 ± 0.05 | 2.45 ± 0.12* | 5.92 ± 0.24** | 10.00 | nd | nd |
| Heinz bodies ($10^3$/μL) | 11 ± 3 | 27 ± 10 | 20 ± 3 | 9 | nd | nd |

Source: NTP 2002a (Table B11).

*$P \leq 0.05$, compared with the vehicle control group by Shirley's test using unrounded data.

**$P \leq 0.01$, compared with the vehicle control group by Dunn's or Shirley's test using unrounded data.

[a]Five blood samples were collected for analysis, but some were lost because of specimen clotting.

[b]No standard error calculated.

nd = No data due to 100% mortality.

**Table 6-7b. Hematology data for female mice dermally exposed to DAAB for 16 days (mean ± SE)**

| Parameter | Vehicle control | Dose (mg/kg b.w.) | | | | |
|---|---|---|---|---|---|---|
| | | 12.5 | 25 | 50 | 100 | 200 |
| n[a] | 4 | 4 | 5 | 5 | 5 | 2 |
| Hematocrit (%) | 46.5 ± 1.5 | 44.0 ± 0.3 | 43.2 ± 0.5 | 39.6 ± 0.8** | 42.9 ± 2.5 | 40.9 ± 0.3 |
| Hemoglobin (g/dL) | 16.5 ± 0.4 | 15.5 ± 0.1 | 15.5 ± 0.1 | 16.4 ± 0.3 | 18.5 ± 1.1 | 17.5 ± 0.6 |
| Erythrocytes ($10^6$/μL) | 9.65 ± 0.32 | 9.10 ± 0.09 | 8.77 ± 0.08 | 8.15 ± 0.13** | 8.94 ± 0.52 | 8.35 ± 0.22 |
| Reticulocytes ($10^6$/μL) | 0.13 ± 0.02 | 0.20 ± 0.02* | 0.35 ± 0.05** | 0.41 ± 0.07** | 0.41 ± 0.07** | 0.39 ± 0.05* |
| Nucleated erythrocytes/100 leukocytes | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| Mean cell volume (fL) | 48.5 ± 0.3 | 48.5 ± 0.3 | 49.4 ± 0.2 | 48.8 ± 0.5 | 48.0 ± 0.3 | 49.0 ± 2.0 |
| Mean cell hemoglobin (pg) | 17.1 ± 0.3 | 17.0 ± 0.1 | 17.7 ± 0.1 | 20.2 ± 0.3** | 20.7 ± 0.1** | 20.9 ± 0.1** |
| Mean cell hemoglobin concentration (g/dL) | 35.5 ± 0.5 | 35.2 ± 0.3 | 36.0 ± 0.1 | 41.6 ± 0.7* | 43.1 ± 0.2** | 42.7 ± 1.6* |
| Methemoglobin (% hemoglobin) | 1.05 ± 0.59 | 1.55 ± 0.13 | 4.68 ± 0.19** | 10.92 ± 0.74** | 15.46 ± 0.47** | 19.00 ± 3.50** |
| Heinz bodies ($10^3$/μL) | 5 ± 5 | 14 ± 3 | 23 ± 7 | 20 ± 2 | 39 ± 15 | 17 ± 17 |

Source: NTP 2002a (Table B11).

*$P \leq 0.05$, compared with the vehicle control group by Shirley's test using unrounded data.

**$P \leq 0.01$, compared with the vehicle control group by Dunn's or Shirley's test using unrounded data.

[a]Five blood samples were collected for analysis, but some were lost because of specimen clotting.

**Table 6-8. Incidences of selected nonneoplastic lesions in male rats dermally exposed to DAAB for 16 days**

| Tissue[a] | Dermal dose (mg/kg) | | | | | |
|---|---|---|---|---|---|---|
| | Vehicle | 12.5 | 25 | 50 | 100 | 200 |
| Skin, application site | | | | | | |
| Hyperplasia | 0[b] | 5** (1.6)[c] | 5** (2.0) | 5** (2.0) | 5** (2.0) | 5** (2.2) |
| Inflammation | 0 | 5** (1.6) | 5** (1.8) | 3** (1.3) | 5** (1.4) | 5** (1.0) |
| Hair follicle hyperplasia | 0 | 4* (1.3) | 5** (2.8) | 5** (3.2) | 5** (3.6) | 5** (4.0) |
| Epidermis, degeneration | 0 | 0 | 5** (2.8) | 5** (3.0) | 5** (2.2) | 5** (2.8) |
| Thymus | | | | | | |
| Atrophy | 0 | 5** (1.0) | 5** (2.6) | 5** (2.4) | 5** (3.0) | 5** (3.0) |
| Lymph node, mesenteric | | | | | | |
| Atrophy | 0 | 2[d] (1.5) | 2 (2.0) | 2 (2.0) | 3 (1.0) | 5** (2.2) |
| Spleen | | | | | | |
| Hematopoietic cell proliferation | 5 (1.2) | 5 (1.0) | 5 (1.2) | 5 (1.6) | 5 (2.0) | 5 (2.0) |

Source: 2002a (Table B8).

*$P \leq 0.05$, **$P \leq 0.01$, compared with the vehicle control group by the Fisher exact test.

[a]5 animals examined microscopically, unless otherwise noted.

[b]Number of rats with lesion.

[c]Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked.

[d]4 animals examined microscopically.

**Table 6-9. Incidences of selected nonneoplastic lesions in female rats dermally exposed to DAAB for 16 days**

| Tissue[a] | Dermal dose (mg/kg) | | | | | |
|---|---|---|---|---|---|---|
| | Vehicle | 12.5 | 25 | 50 | 100 | 200 |
| Skin, application site | | | | | | |
| Hyperplasia | 0[b] | 5** (1.8)[c] | 5** (2.0) | 5** (2.2) | 5** (2.0) | 5** (2.0) |
| Inflammation | 0 | 5** (1.8) | 5** (1.0) | 5** (1.0) | 5** (1.0) | 5** (1.0) |
| Hair follicle hyperplasia | 0 | 5** (2.2) | 5** (3.0) | 5** (3.0) | 5** (2.6) | 5** (3.0) |
| Epidermis, degeneration | 0 | 2 (2.0) | 3 (2.3) | 5** (3.2) | 5** (3.0) | 5** (2.4) |
| Thymus | | | | | | |
| Atrophy | 0 | 4* (1.0) | 5** (1.2) | 5** (2.6) | 5** (2.8) | 5** (3.0) |
| Lymph node, mesenteric | | | | | | |
| Atrophy | 0 | 0 | 0 | 0 | 0 | 4* (1.0) |
| Spleen | | | | | | |
| Hematopoietic cell proliferation | 0 | 4* (1.0) | 5** (1.0) | 5** (1.2) | 5** (2.0) | 5** (1.8) |

Source: NTP 2002a (Table B8).

*$P \leq 0.05$, **$P \leq 0.01$, compared with from the vehicle control group by the Fisher exact test.

[a]5 animals examined microscopically.

[b]Number of rats with lesion.

[c]Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked.

**Table 6-10. Incidences of selected nonneoplastic lesions in male mice dermally exposed to DAAB for 16 days**

| Tissue[a] | Dermal dose (mg/kg) | | | | | |
|---|---|---|---|---|---|---|
| | Vehicle | 12.5 | 25 | 50 | 100 | 200 |
| Skin, application site | | | | | | |
| Epidermis, hyperplasia | 0[b] | 5** (2.0)[c] | 5** (2.0) | 5** (1.8) | 5** (1.4) | 5** (1.0) |
| Hair follicle, hyperplasia | 0 | 5** (1.2) | 5** (1.6) | 5** (1.0) | 5** (1.0) | 2 (2.0) |
| Inflammation | 0 | 5** (1.6) | 5** (1.8) | 5** (1.2) | 5** (1.4) | 5** (1.4) |
| Thymus | | | | | | |
| Atrophy | 0 | 0 | 0 | 5** (3.8) | 5** (4.0) | 5** (4.0) |
| Lymph node, mandibular | | | | | | |
| Atrophy | 0 | 0 | 0 | 3 (2.0) | 4**[d] (2.8) | 5** (2.8) |
| Lymph node, mesenteric | | | | | | |
| Atrophy | 0[d] | 0 | 0[d] | 2 (2.0) | 3 (2.3) | 4*[d] (2.5) |
| Spleen | | | | | | |
| Hematopoietic cell proliferation | 0 | 5** (2.0) | 5** (2.0) | 2 (1.5) | 1 (2.0) | 0 |
| Lymphoid follicle, atrophy | 0 | 0 | 0 | 1 (2.0) | 2 (2.5) | 5** (2.4) |
| Heart | | | | | | |
| Atrium, thrombosis | 0 | 0 | 0 | 2 (2.0) | 5** (3.2) | 5** (3.0) |
| Kidney | | | | | | |
| Bilateral, cortex, renal tubule, necrosis | 0 | 0 | 0 | 0 | 5** (2.0) | 5** (3.0) |
| Liver | | | | | | |
| Necrosis, focal | 0 | 0 | 0 | 0 | 3 (1.3) | 5** (2.6) |

Source: NTP 2002a (Table B12).

*$P \leq 0.05$, **$P \leq 0.01$, compared with the vehicle group by the Fisher exact test.

[a]5 animals examined microscopically, unless otherwise noted.

[b]Number of rats with lesion.

[c]Average severity of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked.

[d]4 animals examined microscopically.

**Table 6-11. Incidences of selected nonneoplastic lesions in female mice dermally exposed to DAAB for 16 days**

| Tissue[a] | Dermal dose (mg/kg) | | | | | |
|---|---|---|---|---|---|---|
| | Vehicle | 12.5 | 25 | 50 | 100 | 200 |
| Skin, application site | | | | | | |
| Epidermis, hyperplasia | 0[b] | 5** (1.2)[c] | 5** (3.0) | 5** (2.6) | 5** (2.4) | 5** (2.0) |
| Hair follicle, hyperplasia | 0 | 5** (1.6) | 5** (3.2) | 5** (3.0) | 5** (3.0) | 5** (3.0) |
| Inflammation | 0 | 5** (2.0) | 5** (1.2) | 5** (2.4) | 5** (2.2) | 5** (2.2) |
| Ulcer | 0 | 0 | 1 (1.0) | 2 (1.5) | 4* (1.3). | 2 (1.0) |
| Thymus | | | | | | |
| Atrophy | 0[d] | 0 | 0 | 4* (3.3) | 5** (3.6) | 3*[d] (4.0) |
| Lymph node, mandibular | | | | | | |
| Atrophy | 0 | 0 | 0 | 0 | 0[d] | 3*[d] (2.0) |
| Lymph node, mesenteric | | | | | | |
| Atrophy | 0 | 0 | 0 | 0 | 1[e] (2.0) | 1[d] (3.0) |
| Spleen | | | | | | |
| Hematopoietic cell prolif. | 0 | 5** (2.0) | 5** (2.0) | 5** (1.4) | 2 (2.5) | 2 (1.5) |
| Lymphoid follicle, atrophy | 0 | 0 | 0 | 0 | 3** (2.7) | 2 (2.5) |
| Heart | | | | | | |
| Atrium, thrombosis | 0 | 0 | 0 | 0 | 1 (1.0) | 2 (2.5) |
| Kidney | | | | | | |
| Bilateral, cortex, renal tubule, necrosis | 0 | 0 | 0 | 0 | 4* (2.5) | 1 (3.0) |
| Bilateral, cortex, renal tubule, dilation | 0 | 0 | 0 | 0 | 0 | 4* (1.5) |
| Liver | | | | | | |
| Necrosis, focal | 0 | 0 | 0 | 0 | 0 | 2 (2.0) |

Source: NTP 2002a (Table B12).

*$P \leq 0.05$, **$P \leq 0.01$, compared with the vehicle group by the Fisher exact test.

[a]5 animals examined microscopically, unless otherwise noted.

[b]Number of rats with lesion.

[c]Average severity of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked.

[d]4 animals examined microscopically.

[e]3 animals examined microscopically.

Several other microscopic findings in mice were considered related to DAAB exposure, many occurring in animals that died early. Atrial thrombosis of the heart was present, seen as a solid coagulum of proteinaceous material and embedded blood cells in the left auricle in all mice that died early. No myocardial changes were evident in either thrombotic hearts of early-death animals or in survivors with increased heart weights. Renal tubule necrosis was observed in early-death male mice and in female mice in the 100-mg/kg group that survived to study termination. Focal liver necrosis was observed in most early-death mice.

#### *6.2.4 Summary of short-term toxicity studies*

Results of these short-term toxicity studies indicate that DAAB is toxic to rats and mice of both sexes at the site of application and systemically. [Based on the small number of animals tested (n = 5), mice are more sensitive to the lethal effects of DAAB than rats, and male mice are more sensitive than females.] Systemic toxicity was most profound in the hematopoietic system of both species.

### 6.3 Potential mechanisms of toxicity and carcinogenicity of DAAB

Although animals were exposed to DAAB for only 16 days in short-term toxicity studies, the results suggest that DAAB has toxic effects characteristic of benzene and aniline; however, most studies with benzene and aniline have used longer exposures. The carcinogenicity of benzene and aniline are described briefly, below, together with possible mechanisms of toxicity and genotoxicity that may be relevant to the discussion of DAAB. As discussed in Section 4.1, Kirby (1947, 1948) reported on dermal exposure studies with DAAB in which mice developed a number of tumors, including squamous papillomas, squamous carcinomas, pulmonary adenomas, and a pulmonary adenocarcinoma.

Like DAAB, benzene and aniline are rapidly absorbed and metabolized and are excreted primarily in urine (McCarthy *et al.* 1985, Mathews *et al.* 1998). As observed in studies of DAAB, benzene was detected unchanged in the breath of rats and mice following administration of benzene by gavage (Mathews *et al.* 1998). The metabolism and excretion of benzene also are dose dependent (Mathews *et al.* 1998). The pathway of benzene metabolism that leads to the formation of muconic acid and hydroquinone is favored at low doses (0.1 mg/kg), whereas at higher doses, the predominant pathways lead to less toxic metabolites. [The dose of DAAB that would be equivalent on a molar basis to a benzene dose of 0.1 mg/kg would be 0.25 mg/kg, which is substantially lower than the dose levels reported above for studies of dermal exposure to DAAB (see Tables 6-6 to 6-10).] The dose- and species-dependent variations in benzene metabolism observed with DAAB previously were reported for benzene (Medinsky *et al.* 1989, Sabourin *et al.* 1989, Mathews and De Costa 1999).

[Analysis of DAAB toxicity is complicated by the fact that it is metabolized to two toxic molecules and also may induce symptoms of toxicity unique to the parent molecule. Analysis also is complicated by the fact that it is difficult to determine the exact internal dose animals received. That is, whereas it is likely that animals exposed dermally received much of the internal dose as a result of grooming, it is impossible to determine

the exact dose received or whether all animals receiving a similar dermally applied dose groomed an equal amount. In any case, conclusions still can be drawn from these short-term studies. As observed in previous studies of benzene, the erythrocyte and lymphoid systems were major targets of DAAB toxicity (IARC 1982a, ATSDR 1997 and references therein). Induction of lymphoid atrophy of the thymus and other lymphoid tissues characteristic of benzene toxicity was obvious in the 16-day study of DAAB toxicity. Symptoms of aniline exposure seen in the study of DAAB include methemoglobin formation, accompanying anemia, increased spleen weights, and regenerative hematopoiesis (CIIT 1982, Short *et al.* 1983, Khan *et al.* 1997).]

[In addition to toxicity induced by benzene and aniline, there is some evidence of toxicity unique to the parent molecule. DAAB apparently was more toxic at the application site than has been reported for equimolar doses of benzene or aniline. Further, though it is difficult to estimate the dose received internally, it appears that even when administered dermally, DAAB may be more toxic than equimolar doses of either benzene or aniline (IARC 1982a, ATSDR 1997 and references therein). The mechanism that accounts for the greater acute toxicity of DAAB has not been determined, but it may be attributable to properties of the parent molecule or to free radicals formed in its metabolism. DAAB induced a greater number of micronuclei than did a combination of equimolar doses of benzene and aniline (see Section 5.3.3, Table 5-1, and Section 6.3.3).]

### *6.3.1 Carcinogenicity of benzene and aniline*

The carcinogenicity of DAAB has not been determined. However, the carcinogenicity of its two metabolites, benzene and aniline, has been evaluated, as reviewed in Section 4.2.

Benzene is a multi-site, multi-species carcinogen that has been extensively reviewed (IARC 1982a, WHO 1993, ATSDR 1997). Chronic inhalation exposure to benzene in rodents has been associated with lymphoma, leukemia, and neoplasia of the Zymbal gland, liver, mammary gland, and nasal cavity. Administration of benzene via oral gavage is associated with neoplasia of the Zymbal gland, oronasal cavity, mammary gland, liver, forestomach, skin, Harderian gland, preputial gland, ovary, and hematopoietic and lymphoreticular systems (NTP 1986). Benzene is listed in the 1st Report on Carcinogens (1980) as *a "known human carcinogen."* Benzene also is listed by IARC (1987a) as Group 1, based on sufficient evidence of carcinogenicity in humans based on numerous case reports and series that "suggested a relationship between exposure to benzene and the occurrence of various types of leukemia."

Aniline-induced carcinogenesis in spleens of rats has been observed in two separate chronic bioassays (NCI 1978, CIIT 1982). In the NCI study, the carcinogenic response in the spleen was specific to the rat and was not observed in $B6C3F_1$ mice exposed concurrently. IARC (1987a) listed aniline as not classifiable as to its carcinogenicity to humans (Group 3) based on inadequate evidence of carcinogenicity in humans and limited evidence of carcinogenicity in experimental animals. The NTP Report on the Metabolism, Toxicity, and Predicted Carcinogenicity of Diazoaminobenzene (2002) concluded that a daily dose of DAAB of 50 mg/kg b.w. given for two years to rats or mice would be equivalent to approximately 25 mg/kg each of benzene and aniline. As

noted in Table 4-1, the cancer effect level for benzene has been reported to be carcinogenic in rats and mice at doses of 25 mg/kg or lower.

### *6.3.2 Mechanisms of toxicity for benzene and aniline*

The toxic effects of DAAB may be related to the toxicity of its metabolites, benzene and aniline. Numerous reports of short-term toxicity induced by benzene describe symptoms of myelotoxicity similar to those observed in the NTP study of DAAB (IARC 1982a, ATSDR 1997 and references therein). Further, as observed in the NTP study of DAAB, rats are more resistant to the toxicity of benzene than are mice. Also, as observed in the 16-week studies of DAAB, aniline is well known for its ability to induce methemoglobinemia (EPA 2000).

Ross (2000) reviewed the literature to address the question of which benzene metabolites are responsible for benzene toxicity and concluded that three different pathways may be responsible. These three pathways involve benzene oxide, the primary oxidation product of benzene; ring-opened reactive metabolites, such as *trans*,*trans*-muconaldehyde; and reactive polyphenols and quinones, such as hydroquinone. Although, as described in Section 6.1.4.2, the metabolite profiles in the blood of rats following oral exposure to DAAB and benzene were qualitatively similar, the DAAB-exposed rats had relatively greater amounts of hydroquinone glucuronide, a major metabolite in one of the putative pathways for benzene toxicity.

Lovern *et al.* (2001) summarized several potential metabolic pathways for benzene toxicity. The ultimate hematotoxin may be 1,4-benzoquinone formed from hydroquinone. 1,4-Benzoquinone is a genotoxin that is a direct alkylating agent, forms DNA adducts, and induces DNA strand breaks. Benzene oxide, the first metabolic product of benzene, also is capable of binding to protein. In addition, the postulated intermediate between 1,2-dihydrodiol and muconic acid, muconaldehyde, can induce DNA-protein crosslinks. Another metabolite of benzene, catechol (or 1,2-dihydroxybenzene), can be oxidized to 1,2-catechol quinone, which can form depurinating DNA adducts (Cavalieri *et al.* 2002). Golding and Watson (1999) concluded that the significance of DNA adduct formation in benzene-induced human leukemogenesis was unknown and suggested that other reactions of benzene metabolites with enzymes such as topoisomerase II also could play a role in the toxicity of benzene. Goldstein and Witz (2000) also reviewed the evidence for pathways of toxicity of benzene and concluded that “the effect of benzene is likely to be exerted through the action of multiple metabolites on multiple endpoints through multiple biological pathways.”

IARC (1982b) concluded that *N*-hydroxylation of aniline is probably related to the methemoglobinemia caused by aniline in humans. In rats and mice administered aniline, the predominant urinary metabolites were, respectively, 4-acetamidophenyl sulfate and 4-acetamidophenyl glucuronide (McCarthy *et al.* 1985). Following exposure to DAAB, these were the primary aniline metabolites detected in urine of mice, and 4-acetamidophenyl sulfate was the only aniline metabolite observed in the urine of rats. Mice also excreted three additional metabolites associated with aniline (Table 6-5).

### *6.3.3 Genotoxicity of DAAB, benzene, and aniline*

The vast majority of organic chemicals identified as human carcinogens induce mutations in *S. typhimurium* and micronuclei and chromosomal aberrations in rodent bone marrow (Shelby 1988, Shelby and Zeiger 1990). Ress *et al*. (2002) demonstrated that DAAB, benzene, and aniline induce micronuclei in mice (see Section 5.3.3). The authors also noted that DAAB induced more micronuclei in mice than did equimolar doses of benzene or a mixture of benzene and aniline, an effect that they suggested could be due to formation of the phenyl radical during metabolism of DAAB. Aniline was considered a weak inducer of micronuclei. Benzene induced micronuclei at doses ranging from 10 to 40 mg/kg b.w., similar to the lowest doses associated with carcinogenicity (25 to 50 mg/kg b.w., as reported in Section 4.2). Similarly, aniline induced micronuclei at 23 and 470 mg/kg b.w. and tumors at 30 to 200 mg/kg b.w. Therefore, as concluded by Ress *et al.* (2002), the genotoxicity of DAAB in mouse bone marrow and its similarity to that of benzene could support a prediction of carcinogenicity for DAAB.

## 6.4 Summary

DAAB was almost completely absorbed from the gastrointestinal tract but sparingly absorbed from skin. For each route of administration, the absorbed portion of the dose was rapidly metabolized and excreted primarily in urine. Within 24 hours, approximately 60% of an oral dose of radiolabeled DAAB was accounted for as metabolites of benzene or aniline in the urine of rats and mice, and total recoveries of metabolites were quantitatively similar to the predicted values of 40% benzene and benzene metabolites and 47% aniline and aniline metabolites. The studies described above demonstrated that in rats and mice, DAAB is quantitatively metabolized to benzene, a known human and animal carcinogen, and to the rat carcinogen, aniline. In addition, i*n vitro* studies in human liver samples also demonstrated that DAAB could be cleaved to yield metabolites of benzene and aniline. Symptoms observed in animals administered DAAB are similar to those that would be anticipated in animals exposed to a combination of benzene and aniline. Skin painting studies with DAAB in mice resulted in several types of tumors, including squamous papilloma, squamous carcinoma, pulmonary adenoma, and pulmonary adenocarcinoma. DAAB also was observed to induce micronuclei in mouse bone marrow at molar doses similar to micronucleus-inducing doses of benzene and aniline. DAAB metabolism also results in the formation of a reactive phenyl radical, which could account for an additional risk of toxicity and/or carcinogenicity. Evidence for the possible involvement of the phenyl radical in DAAB mutagenicity is seen in the fact that DAAB causes mutations in *S. typhimurium* with metabolic activation, whereas benzene and aniline do not.

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# Appendix A: NTP TR 73 (2002). NTP Report on the Metabolism, Toxicity, and Predicted Carcinogenicity of Diazoaminobenzene (CAS No 136-35-6). PP A-1 – A-87.